Volume 56 MAY, 1955 Number 5

DOES NOT CIRCULATE
UNIVERSITY OF MICHIGAN
MAY 17 1955
MEDICAL LIBRARY

ENDOCRINOLOGY

FIELD, JAMES B.
Prevention of Adrenal Cortical Hypertrophy in Diabetic Rats by the Use of Insulin 499

MOOKERJEA, SAILEN AND D. P. SADHU
Metabolism of Acetic Acid in Kidney and Liver Slices of the Hypothyroid Rats ... 507

LAZO-WASEM, E. A. AND M. X. ZARROW
The Conversion of Desoxycorticosterone Acetate to a Progesterone-like Substance 511

SELYE, HANS
Effect of Corticoids Upon the Development and Toxicity of Transplantable Croton-Pouch Tumors 516

SAFFRAN, MURRAY AND ANDREW V. SCHALLY
In Vitro Bioassay of Corticotropin: Modification and Statistical Treatment 523

HAUSBERGER, FRANZ X. AND ANDREW J. RAMSAY
Steroid Diabetes in Guinea Pigs 533

MASSON, G. M. C., EDWIN R. FISHER, A. C. CORCORAN AND IRVINE H. PAGE
Effects of Renin in Rats Treated with Methylandrostenediol 541

NICHOLLS, DORIS AND R. J. ROSSITER
The Role of the Pituitary and Thyroid Glands in the Phosphorus Metabolism of the Adrenal Gland During Cold Stress 547

GOLDBERG, R. C., J. WOLFF AND R. O. GREEP
The Mechanism of Depression of Plasma Protein Bound Iodine by 2,4 Dinitrophenol 560

SMITH, ELIZABETH KNAPP
Interrelationships of Anterior Pituitary and Adrenal Cortex in the Rat Ovarian Hyperemia Reaction 567

STURKIE, PAUL D.
Effects of Gonadal Hormones on Blood Sugar of the Chicken 575

PERRY, WILLIAM F. AND HELEN F. BOWEN
The Effect of Growth Hormone on Lipogenesis in Intact and Adrenalectomized Rats 579

NADEL, E. M., A. S. MULAY AND L. D. SASLAW
On the Failure of Glycogen Deposition in the Livers of Scorbutic Guinea Pigs ... 584

SCOW, ROBERT O. AND MONTE A. GREER
Effect on the Thyroid Gland of Experimental Alteration of the Level of Circulating Thyroxine in Mice with Heterotopic Pituitaries 590

CHANG, CHIH-YE AND EMIL WITSCHI
Independence of Adrenal Hyperplasia and Gonadal Masculinization in the Experimental Adrenogenital Syndrome of Frogs 597

NOTES AND COMMENTS

LIPNER, H. J., BILLIE P. WAGNER AND HAROLD P. MORRIS
The Effect of Propylthiouracil on the Growth and Thyroid: Serum Iodide Ratio of Mouse Thyroid Autotransplants 606

BROWN-GRANT, K.
Inhibition of the Release of Thyroidal Radioiodine in the Rat by Cortisone 607

BARUCH, HANS AND I. L. CHAIKOFF
Lipogenesis and CO_2 Formation from Acetate in the Liver of the Hypophysectomized Rat 609

COWIE, A. T. AND J. S. TINDAL
Maintenance of Lactation in Adrenalectomized Rats with Aldosterone and 9α-Halo Derivatives of Hydrocortisone 612

KNOBIL, ERNEST AND MELVIN J. FREGLY
Effect of Ascorbic Acid on the Adrenal Gland after Hypophysectomy and after Exposure to Cold 614

KOSTYO, JACK L. AND SAMUEL L. LEONARD
Glycogenolytic Effects of Epinephrine and Posterior Pituitary Extracts on the Uterus and Skeletal Muscle 616

THE ENDOCRINE SOCIETY 619

THE SOCIETY FOR ENDOCRINOLOGY 620

ISSUED MONTHLY FOR THE ENDOCRINE SOCIETY
BY CHARLES C THOMAS, PUBLISHER,
SPRINGFIELD, ILLINOIS

Annual Subscription $13.00 Single Copies $1.50

For an Estimated 4 out of 1,000,000 People More Than 3 Years of Pharmaceutical Research

In 1855 Addison first delineated the classical clinical picture of adrenocortical insufficiency. Today, as then, Addison's disease is a rare (an estimated 4 per 1,000,000 population) but serious affliction.

The synthesis of desoxycorticosterone was an important step in the control of adrenocortical insufficiency. However, the easy solubility and consequent short activity of this potent hormone steroid imposed severe limitations to its use.

CIBA, constantly in the forefront of endocrine research, spent more than 3 years developing a desoxycorticosterone derivative that would be more slowly absorbed and provide more prolonged activity. The result was **Percorten trimethylacetate.**

A single intramuscular injection of **Percorten trimethylacetate** maintains the patient with adrenal insufficiency for about a month. With this new long-acting hormone, the unpredictable and inconvenient process of surgical implantation of desoxycorticosterone pellets is no longer necessary.

The introduction of **Percorten trimethylacetate** marks a significant advance in therapy of adrenocortical insufficiency. Its development is typical of CIBA research—the persistent, painstaking quest for better pharmaceutical specialties, whether for the rare or the widespread diseases.

Supplied: Multiple-dose Vials, 4 ml., containing 25 mg. per ml. as an aqueous microcrystalline suspension for intramuscular use only.

Percorten® trimethylacetate (desoxycorticosterone trimethylacetate CIBA)

C I B A
SUMMIT, N. J.

4/2095M

ENDOCRINOLOGY

VOLUME 56 MAY, 1955 NUMBER 5

Copyright 1955 by the Endocrine Society

PREVENTION OF ADRENAL CORTICAL HYPERTROPHY IN DIABETIC RATS BY THE USE OF INSULIN[1]

JAMES B. FIELD

Departments of Pediatrics and Medicine, Massachusetts General Hospital, Boston, Massachusetts

SEVERAL recent papers (Bennett and Koneff, 1946; Applegarth, 1949; Eranko, 1951) have reported enlargement and increased function of the adrenal cortex of rats made diabetic with alloxan. Bennett and Koneff (1946) felt this hypertrophy was probably due to the altered metabolism of the diabetic animal rather than the direct toxic effect of alloxan. Eranko (1951), however, suggested the changes might reflect the increased secretion of insulin following the injection of alloxan.

This observation of increased adrenal cortical function is contrary to the findings of Field and Marble (1951) who noted diminished adrenal cortical activity in some diabetics. This apparent contradiction might depend on whether an attempt is made to regulate the diabetes with insulin and thereby reduce the stress of an uncontrolled metabolic state. The present study was undertaken to determine whether insulin would prevent the adrenal cortical hyperactivity of diabetic rats using histochemical appearances as well as adrenal weights as a basis of comparison.

METHODS AND MATERIALS

Parallel experiments lasting 72 hours and three weeks were done using male rats of the Long-Evans strain weighing approximately 150 gm. The rats were divided into three groups, each containing five to eight animals—a non-diabetic control group; an untreated diabetic group; and an insulin treated diabetic group. Diabetes was produced by the intraperitoneal injection of 250 mg. of alloxan per Kg. after the animals had been fasted for 24 hours. However, in the group of diabetic rats not given insulin in the 21 day experiment, the dose was reduced to 200 mg. per Kg. because of the difficulty keeping these rats alive for three weeks if they were given the larger dose of alloxan. The animals were permitted to eat and drink *ad lib.* of a diet consisting of Purina laboratory chow. At the end of the experiment they were sacrificed by a sudden blow on the head.

Received August 3, 1954.

[1] This investigation was supported by a grant from Eli Lilly and Company.

During the 72-hour experiment regular crystallin insulin was given subcutaneously three times a day. The dose was based on blood sugar determinations twice and a Benedict qualitative urine sugar test the other time. These were so arranged that insulin was given every 7 to 8 hours. The second blood sugar was taken approximately three hours after the first dose of insulin was given and thus served as a check on its effect. On the average, one unit of insulin was given for a blood sugar over 500 mg. %, $\frac{3}{4}$ unit for a figure over 400 mg. %, $\frac{1}{2}$ unit for levels over 300 mg. % and $\frac{1}{4}$ unit for values over 150 mg. %. As can be seen from Table 1, which represents an average protocol, the diabetes was not always well controlled, the animals tending to remain hyperglycemic with varying degrees of glycosuria.

TABLE 1. PROTOCOL OF RAT NUMBER 8

Day	Time	Blood sugar	Urine test	Insulin dose	Weight
1	9:00 A.M.		Green		150.0 gms.
2	10:00 A.M.	748 mg. %	Red-Orange		
	1:00 P.M.			$1\frac{1}{4}$ unit	
	4:00 P.M.	101.5 mg. %	Red-Orange		
	11:00 P.M.		Olive-Green	$\frac{1}{2}$ unit	
3	10:00 A.M.	510 mg. %	Olive-Green		
	12:00 noon			1 unit	
	4:00 P.M.	153.3 mg. %	Brown		
	8:00 P.M.		Green	$\frac{1}{4}$ unit	
4	2:00 A.M.		Green	$\frac{1}{4}$ unit	
	9:00 A.M.		Green		135.2 gms.

In the three week experiment, the insulin requirement was determined by the above method for the first three days. Thereafter the urine sugar was tested three times a day and the previously determined insulin dosage modified accordingly. In every case the daily amount of insulin increased during the three weeks without much change in the urine sugar tests. Occasional blood sugars were done three hours after an insulin injection as a check on the urine control. Table 2 is the protocol of one of these animals and again indicates the difficulty of maintaining good diabetic regulation. The blood sugar determinations were done by the method of Folin (1932) on tail blood.

At autopsy the adrenal glands were weighed immediately on a torsion balance and then fixed in 10% neutral formalin. Sections were stained with sudan black B and by the Schultz cholesterol method to reveal their lipid and cholesterol content.

RESULTS

Table 3 summarizes the results of the 72-hour experiment. The blood sugars of the non-diabetic animals ranged from 76 mg. % to 126 mg. % while those of the untreated diabetic animals varied from 516 mg. % to 1012 mg. %. The initial blood sugars of the treated diabetic rats 24 hours after alloxan injection and before insulin administration were from 238 mg. % to 748 mg. %. Four of the eight animals in the last group had blood sugars ranging from 509 mg. % to 1020 mg. % sometime during the experiment even though they were receiving insulin. This is an indication that the severity of the diabetes was roughly equivalent in both groups of animals, and also illustrates the difficulty involved in regulating the diabetes.

The untreated diabetic rats lost weight during the experiment while the

TABLE 2. PROTOCOL OF RAT NUMBER 51

Day	Time	Blood sugar	Urine test	Insulin dose	Weight
1	9:00 A.M.		Green		160.5 gms.
2	3:00 P.M.	628 mg. %			
	7:00 P.M.			1 unit	
3	1:00 A.M.		Brown	$\frac{3}{4}$ unit	
	8:00 A.M.	510 mg. %	Orange-Brown		
	12:00 noon		Brown	$\frac{3}{4}$ unit	
	3:00 P.M.	93 mg. %	Brown		
	7:00 P.M.			$\frac{1}{2}$ unit	
4	1:00 A.M.		Brown	$\frac{3}{4}$ unit	
	10:00 A.M.		Brown	$\frac{3}{4}$ unit	
	4:00 P.M.		Brown	$\frac{3}{4}$ unit	
	11:00 P.M.		Orange-Brown	1 unit	
7	7:00 A.M.		Orange-Brown	$1\frac{1}{4}$ unit	
	2:00 P.M.		Orange-Brown	$1\frac{1}{4}$ unit	
	10:00 P.M.		Orange-Brown	$1\frac{1}{2}$ unit	
10	8:00 A.M.		Brown	1 unit	
	2:00 P.M.		Mustard	1 unit	
	10:00 P.M.		Brown	$1\frac{1}{4}$ unit	
13	8:00 A.M.		Orange-Brown	$1\frac{1}{4}$ unit	
	3:00 P.M.		Orange	$1\frac{1}{2}$ unit	
	11:00 P.M.		Orange-Brown	$1\frac{1}{2}$ unit	
15	8:00 A.M.		Mustard	$1\frac{1}{2}$ unit	
	3:00 P.M.		Brown	$1\frac{1}{2}$ unit	
	11:00 P.M.		Brown	$1\frac{1}{2}$ unit	
17	8:00 A.M.		Brown	$1\frac{3}{4}$ unit	
	3:00 P.M.		Brown	$1\frac{3}{4}$ unit	
	10:00 P.M.		Brown	$1\frac{3}{4}$ unit	
19	8:00 A.M.		Brown	2 units	
	3:00 P.M.		Mustard	$1\frac{3}{4}$ unit	
	10:00 P.M.		Orange-Brown	2 units	
20	8:00 A.M.		Orange-Brown	2 unist	
	3:00 P.M.		Brown	2 units	
	6:00 P.M.	199 mg. %	Orange-Brown		
	10:00 P.M.		Mustard	2 units	
21	8:00 A.M.		Brown	$2\frac{1}{4}$ units	198.5 gms.

non-diabetic animals gained. The rats given insulin also tended to lose weight, although not as much as the untreated diabetic animals. Because of this variation in body weight, the ratio of the adrenal weight to 100 gm. of initial body weight was used as a basis of comparison. Although there was some overlap in all three groups there was no significant difference between the ratios of the insulin treated and normal rats. However, the adrenal weight ratios of the untreated diabetic animals were significantly larger than the other two groups. The ratio for the control group was 25.4

TABLE 3. RESULTS OF 72 HOUR EXPERIMENT

No. animals	Initial blood sugar, mg. %	Initial weight, gm.	Final weight, gm.	Weight change, gm.	Adrenal weight, mg.	Adrenal/ 100 gm. I. W.	Adrenal/ 100 gm. F. W.
8*	412 ±162	137.7±12.5	131.2±14.6	− 6.5±6.1	40.4±10.6	29.2± 6.2	30.7± 6.6
8†	699.5±142.9	132.9±23.3	111.4±19.2	−21.5±9.4	53.6±14.7	40.8±10.6	48.5±12.8
5‡	98.4± 20.9	140.8±19.1	148.7±23.8	7.9±7	35.2± 6.6	25.4± 5	22.8± 4.6

* Insulin treated diabetic animals.
† Untreated diabetic animals.
‡ Non-diabetic control animals.
The differences between the ratios of adrenal weight to 100 gm. of initial rat weight of the untreated diabetic animals to the insulin treated and non-diabetic groups are statistically significant. P is between .02 and .05 in the former and .01 in the latter animals.

mg./100 gm. initial rat weight; for the insulin treated rats it was 29.2; and for the untreated diabetic animals 40.8. This seems to indicate that adrenal hypertrophy can be prevented by using insulin to control the diabetes.

Grossly the adrenals from the untreated diabetic rats were reddish brown and often hemorrhagic as compared to the pale yellow color of the glands from the insulin treated and normal rats. Histologic examination of the adrenals also suggests that the increased adrenal cortical function can be prevented by regulating the diabetes with insulin. Figure 1 is a control adrenal, showing abundant lipid in the glomerulosa and in the outer fasciculata. The Schultz cholesterol test gave an intense reaction in all lipid droplets. A representative gland from the untreated diabetic group is shown in Figure 2. It was enlarged and contained virtually no lipid in the glomerulosa or fasciculata; the Schultz reaction was negative except in an occasional glomerulosa cell. In contrast, the insulin treated animals (Fig. 3) had adrenals which were somewhat enlarged but contained only slightly more lipid than normal in the fasciculata. The appearance of the gland in Figure 3 is somewhat deceptive in that it looks as large as that in Figure 2. It should be noted that it came from a larger animal and weighed 56 mg. as compared to the 42.3 mg. of the gland in Figure 2. However, histologi-

---

Frozen sections of adrenal glands, stained with sudan black B for 1 minute, photographed with a blue filter (Wratten H), ×80.

FIG. 1. Rat #28, control for 3-day experiment. Adrenal weight 31.1 mg.; relative weight 18.3 mg./100 gms. initial body weight. Lipid droplets are abundant in the cells of the zona glomerulosa, absent from the subglomerulosal transitional zone, and abundant in the outer portion of the zona fasciculata. The Schultz reaction for cholesterol showed the same pattern of intensity.

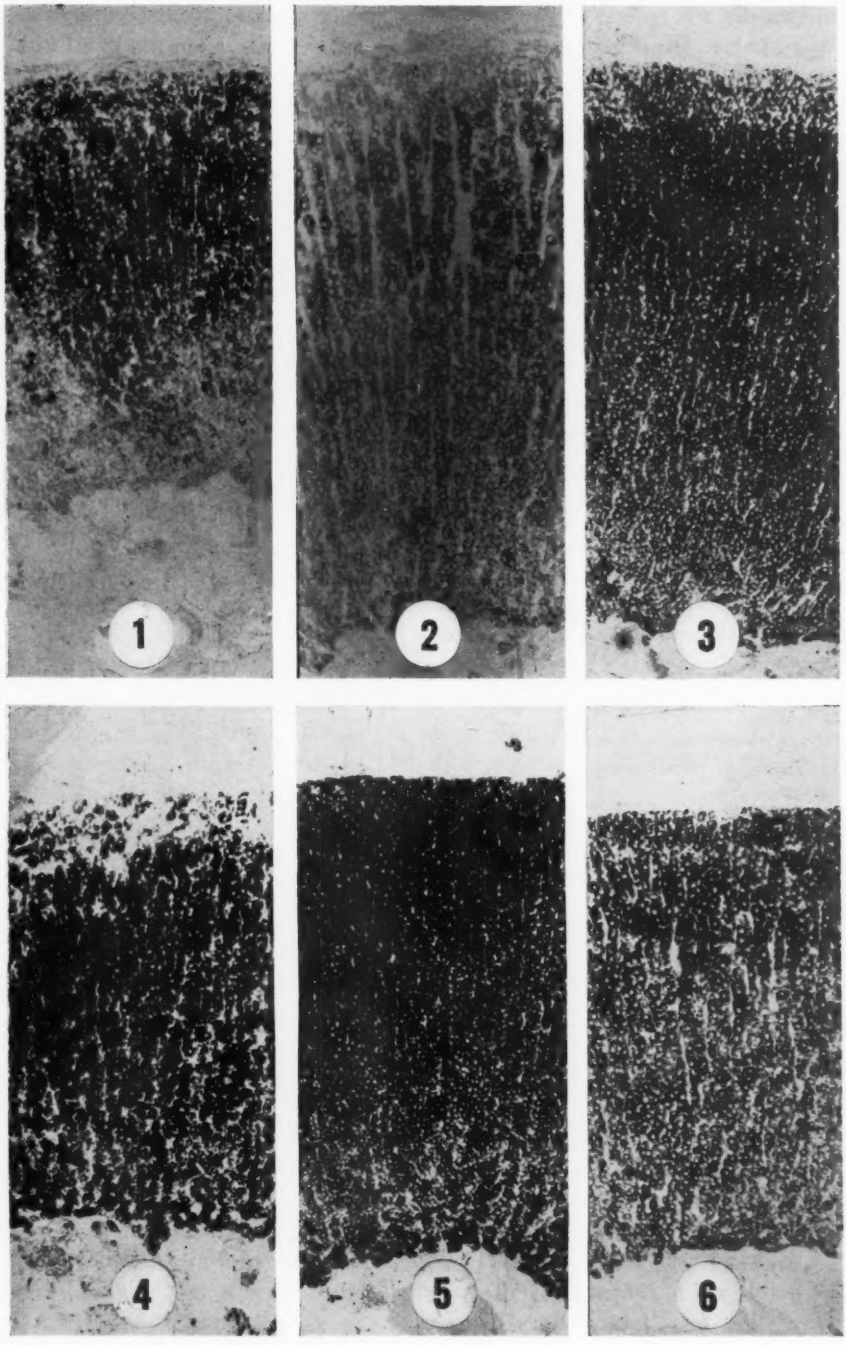
FIG. 2. Rat #19, alloxan diabetes for 3 days. Adrenal weight, 42.3 mg.; 37.1 mg. %. The adrenal cortex is enlarged and shows virtually complete depletion of lipids from both the zona glomerulosa and the zona fasciculata. The Schultz reaction was negative except in occasional glomerulosa cells.

FIG. 3. Rat #2, alloxan diabetes controlled with insulin, 3 days. Adrenal weight 56 mg.; 39.7 mg. %. The cortex is enlarged; free lipid is greatly depleted from the zona glomerulosa but is present in increased concentration in the zona fasciculata. The Schultz reaction showed the same distribution as the sudanophilic lipids, but was weaker than in Rat #28.

FIG. 4. Rat #86, control for 21-day experiment. Adrenal weight 43 mg.; 25.1 mg. %. Normal appearance. The Schultz reaction was moderately intense in the glomerulosa and outer half of the fasciculata.

FIG. 5. Rat #61, alloxan diabetes for 21 days. Adrenal weight 55.7 mg.; 37 mg. %. Somewhat broadened cortex showing marked accumulation of lipid in both the zona glomerulosa and the entire zona fasciculata. No transitional zone. The Schultz reaction was only moderate in intensity but uniformly distributed throughout the cortex.

FIG. 6. Rat #52, alloxan diabetes controlled with insulin, 21 days. Adrenal weight 45.8 mg.; 24.4 mg. %. The adrenal cortex appears indistinguishable from normal, and also showed a normal distribution and concentration of Schultz-positive material.


1
2
3
4
5
6

cally it did not appear to be as hyperactive as the gland from the untreated diabetic rat. Hypertrophy of the adrenal cortex with depletion of its lipid content is associated with markedly increased cortical secretion (Greep and Deane, 1945; Miller, 1950). Since the glands from the untreated diabetic animals were depleted of lipid, they were presumably functioning more actively than those from the insulin treated rats where the lipid was slightly increased. The significance of this slight increase in lipid is difficult to assess (Miller, 1950). It is of interest to note both the untreated and insulin treated diabetic animals showed marked depletion of lipid from the glomerulosa. Since these changes in the glomerulosa can be seen following kidney damage (Olson and Deane, 1949), they might reflect the renal toxicity of alloxan (Bailey *et al.*, 1945). Insulin administration would not be expected to prevent this effect.

The results of the 21-day experiment are summarized in Table 4. As pointed out by Eranko (1951) the changes are not as clear cut as those of the acute experiment. The non-diabetic animals had blood sugars between 101 mg. % and 155 mg. % while those of the untreated group ranged from

TABLE 4. RESULTS OF 21 DAY EXPERIMENT

No. animals	Initial blood sugar, mg. %	Initial weight, gm.	Final weight, gm.	Weight change, gm.	Adrenal weight, mg.	Adrenal/ 100 gm. I. W.	Adrenal/ 100 gm. F. W.
5*	495±175	167.4±26.2	197.9± 7.1	30.5±30.1	49.9± 8.9	30.2±4.7	25.2± 4.1
6†	445±116.7	143.4±10.3	123 ±18.6	−20.4±20.4	46.4±12.3	32.3±6	39.2±13.3
6‡	117± 19.9	150.1±12.1	230.1±19.3	80 ±15.1	48.3± 6.5	32.3±4.5	21.0± 1.8

* Insulin treated diabetic animals.
† Untreated diabetic animals.
‡ Non-diabetic control animals.

302 mg. % to 600 mg. %. The initial blood sugars in the treated diabetic animals before insulin administration were from 296 mg. % to 675 mg. % and four of these five animals had levels over 550 mg. % at some time during the experiment. Thus it might appear that the diabetes was more severe in the insulin treated rats than in the untreated diabetic animals. This might be a reflection of the slightly smaller dose of alloxan used in the latter animals because of the difficulty in keeping them alive. During the experiment all of the non-diabetic animals continued to grow and gain weight while five of the six untreated diabetic rats lost weight. The sixth animal gained only 3 gm. in the 21 day period. All of the insulin treated animals gained weight, although not as much as did the normals. Because of the wide variation in the final weights of the animals, it is very difficult to properly evaluate the changes in the size of the adrenals. There was no significant difference between the ratios of adrenal weight to 100 gm. of initial rat weight in any of the three groups of animals. Furthermore, the histologic appearance of the adrenal glands reveals only slight differences. The non-diabetic adrenals were completely normal and contained

abundant lipid throughout the cortex (Fig. 4). The untreated diabetic rats (Fig. 5) showed some increase in the lipid content of the glomerulosa as well as the fasciculata. Although a few of the glands of the insulin treated animals also showed a slight increase in their lipid content, the majority of them (Fig. 6) did not differ from the controls.

DISCUSSION

In the 72-hour experiment the adrenal hypertrophy of diabetic rats was prevented by using insulin. This observation is in accord with that of Rose (1951), who was able to prevent most of the adrenal hyperactivity as measured by adrenal weights and the ascorbic acid and cholesterol content of the glands. The marked depletion of lipid and cholesterol, reflecting greatly increased adrenal cortical function, was seen only in the untreated diabetic animals. The slight differences between the non-diabetic and insulin treated animals can probably best be explained by the fact that the diabetes in the insulin treated rats was not perfectly regulated.

Since alloxan was used in both the insulin treated and untreated diabetic animals it is unlikely that its toxic effect was directly responsible for the adrenal enlargement in the latter rats. The transient hypoglycemia produced by alloxan might have been an initial stimulus to the adrenal, but it was not sufficiently prolonged to counterbalance the effect of insulin in preventing adrenal hypertrophy. The stress of uncontrolled diabetes rather than the diabetic state per se is a more probable explanation for the enlarged adrenals in the untreated diabetic animals. The animals given insulin were still diabetic but their metabolic defect was partially corrected by insulin administration.

The results of the 21-day experiment are inconclusive. There was no significant difference between the ratios of weights of the adrenals to 100 gm. initial rat weight or the histologic appearance of the adrenals in all three groups of animals.

SUMMARY

In an acute experiment lasting 72 hours adrenal cortical hypertrophy was prevented in diabetic rats by regulating the diabetes with insulin. There was no significant difference between the ratio of adrenal weights to 100 gm. initial animal weight of the insulin treated animals and the normal animals while the difference between these two groups and the untreated diabetic rats was statistically significant. Only the adrenals of the untreated diabetic rats showed depletion of lipids and cholesterol, indicating marked adrenal cortical activity.

When the experiment was prolonged to three weeks, the results were inconclusive. Lipid and cholesterol were slightly increased in the untreated diabetic rats whereas the insulin treated animals had adrenals which were

almost indistinguishable from the control rats. There was no difference in the ratio of the adrenal weights to 100 gm. of initial body weight in these three groups.

Acknowledgment

The author wishes to thank Dr. Helen W. Deane for the histochemical studies and Drs. Janet McArthur, Richard Cohen, and Allan M. Butler for their helpful suggestions and criticisms.

REFERENCES

APPLEGARTH, A.: *Endocrinology* **44**: 197. 1949.
BAILEY, C. C., O. T. BAILEY AND R. S. LEACH: *Bull. New England Med. Center* **7**: 59. 1945.
BENNETT, L. L. AND A. A. KONEFF: *Anat. Rec.* **96**: 1. 1946.
ERANKO, O.: *Acta Endocrinol.* **6**: 97. 1951.
FIELD, J. B. AND A. MARBLE: *Proc. Soc. Exper. Med. and Biol.* **77**: 195. 1951.
FOLIN, O.: *New England J. Med.* **206**: 727. 1932.
CREEP, R. O. AND H. W. DEANE: *Ann. New York Acad. Sc.* **50**: 596. 1949.
MILLER, R. A.: *Am. J. Anat.* **86**: 405. 1950.
OLSON, R. E. AND H. W. DEANE: *J. Nutrition* **39**: 31. 1949.
ROSE, S.: *Australian J. Exper. Biol. and M. Sc.* **29**: 469. 1951.

METABOLISM OF ACETIC ACID IN KIDNEY AND LIVER SLICES OF THE HYPOTHYROID RATS

SAILEN MOOKERJEA[1] AND D. P. SADHU

Department of Physiology and Nutrition, Bengal Veterinary College, Calcutta, India

HYPOTHYROIDISM causes depression of various enzymatic process and O_2 consumption of the tissues (Boreld and Holmgreen, 1948; Tipton and Nixon, 1946). It also induces hypercholesterolemia, rise in liver cholesterol (Handler, 1948) and caracass cholesterol (Kennely and Maynard, 1952). A rise of volatile fatty acids (which include 90% acetic acid), acetone bodies and cholesterol with a depression of succinic and lactic dehydrogenase activity have been observed by the authors (1954) in the tissues of hypothyroid rats even when the effects of unequal food intake was neutralised.

Acetate and acetoacetate are the basic materials for cholesterol synthesis (Brady and Gurin, 1951) and they are oxidized through the tricarboxylic acid cycle (Krebs, 1943; Krebs and Jhonson, 1937). Besides liver, kidney oxidises acetates very rapidly, forms acetoacetate (Medes *et al.*, 1945a, 1945b) and also synthesises cholesterol (Srere, 1950). So all these facts make the metabolism of acetic acid a logical choice for study in liver and kidney of hypothyroidism induced rats to throw further light into the mechanism of cholesterol synthesis from its precursors.

EXPERIMENTAL

Growing male albino rats maintained on stock laboratory diet were divided into groups of eight each and hypothyroidism induced in one group (H) by including 0.16% methylthiouracil in its diet. Paired feeding technique was adopted to avoid any effects of unequal consumption of food.

Animals were sacrificed after 6 to 7 weeks. O_2 consumption of liver and kidney slices in "Krebs-Ringer Phosphate" was measured with (A) or without (C) 0.08M sodium acetate in Warburg vessels at 38° C. in 100% oxygen. Acetoacetic acid was determined by Edson's (1935) method. Final readings were recorded after attainment of constancy, usually 30–45 minutes at 38° C. Q_{Acac} has been expressed as by Edson (1935), being the μl of CO_2 acetoacetic acid formed per mg. dry weight of tissue per hour.

Free and ester cholesterol in blood and tissues were estimated by the

Received September 15, 1954.

[1] Present address: Department of Biochemistry, Nagpur University, Nagpur, India.

method of Sobel and Mayers (1945) as modified by the authors (1955, in press) to make it suitable for the tissues.

RESULTS

Table 1 shows the average weight and food intake of the rats in 1–2 and 6–7 weeks period of the experiment. H group was required to be maintained with pair-feeding from the 3rd/4th week.

TABLE 1. AVERAGE BODY WT. AND FOOD INTAKE WITH ± STANDARD ERROR OF THE NORMAL (N) AND HYPOTHYROID (H) RATS IN 1–2 AND 6–7 WKS. PERIOD OF THE EXPERIMENT

Condition of the animals	1–2 weeks		6–7 weeks	
	Average body wt. in gm.	Food intake per 100 gm. body wt. in gm.	Average body wt. in gm.	Food intake per 100 gm. body wt. in gm.
N	75 ± 3.8	11.5 ± 1.1	106 ± 4.1	6.5 ± 1.4
H (with 0.16% METU in the diet)	72 ± 4.1	11 ± 2	123 ± 3.6	6.5 ± 1.8

Table 2 shows the increase in cholesterol values of liver, kidney and blood of the hypothyroid rats. The table also shows decreased QO_2 and increased Q_{Acac} in hypothyroid liver and kidney slices compared to the normals. The column of percentage of increase shows that of increment of QO_2 by adding acetate, since substrate was also less in hypothyroid animals. Though the Q_{Acac} values in normal kidney slices was almost insignificant but in hypothyroid animals the Q_{Acac} in both C and A were increased considerably.

DISCUSSION

Acetic acid stands at a key position of carbohydrate and lipid metabolism. As an acetic acid derivative, i.e. acetyl COA, the carbohydrates and fats are oxidized to CO_2 and H_2O through the tricarboxylic acid cycle.

The results in Table 2 show that in condition of hypothyroidism, there is a depression of acetate oxidation in liver and kidney slices, specially in the latter, which normally oxidises acetate at a very rapid rate. Formation and accumulation of more acetoacetate and significant increase of cholesterol is also evident in such condition. A condition of continued depression of acetate oxidation in hypothyroidism may lead to a diversion of unoxidised acetate for cholesterol synthesis. Formation and accumulation of more acetoacetate indicate that finally cholesterol is being synthesised from both the precursors. Recent investigations show that the rate of cholesterol synthesis by surviving rat liver is markedly greater at lower pH (Curran, 1953). So a reduction in intracellular pH for the accumulation of acetoacetate in hypothyroidism may play role in cholesterolegene-

TABLE 2. FREE AND ESTER CHOLESTEROL VALUES OF BLOOD, KIDNEY AND LIVER OF NORMAL (N) AND HYPOTHYROID (H) RATS WITH THEIR RATIOS (F/E) AND Q_{O_2}* AND Q_{Acac}† VALUES OF THE LIVER AND KIDNEY SLICES WITH THEIR INCREASE IN %

Condition and number of the animals	Liver						Kidney						Blood		
	Cholesterol, mg./100 gm. wet wt.			Substrate	Q_{O_2}	Q_{Acac}	Cholesterol, mg./100 gm. wet wt.			Substrate	Q_{O_2}	Q_{Acac}	Cholesterol, mg./100 gm. wet wt.		
	Free	Ester	F/E				Free	Ester	F/E				Free	Ester	F/E
N, 8	238 ± 19	69 ± 11	3.4	"C"[1]	− 9.1 ± 1.2	0.96 ±0.05	412 ± 22	87 ± 7	4.7	"C_1"	−17.9 ± 2.8	0.21 ±0.01	56 ± 7	74 ± 8	0.7
				"A"[2]	−11.9 ± 1.8	1.31 ±0.08				"A"	−24.5 ± 2.1	0.34 ±0.05			
Increase in %					30%	36%					36.9%	61%			
H, 8 (With 0.16% METU in the diet).	251 ± 31	90 ± 12	2.8	"C"	− 7.2 ±1.3	1.21 ±0.2	486 ± 26	113 ± 11	4.3	"C"	−13.9 ± 2.1	0.71 ±0.06	84 ± 6	164 ± 13	0.5
				"A"	− 8.1 ±1.5	2.09 ±0.6				"A"	−17.8 ± 2.2	1.67 ±0.1			
Increase in %					12.2%	72%					28%	121%			

Results are given as average value ± STANDARD error.
* Q_0 = ml. O_2 uptake per mg. dry wt. of tissue per hour.
† Q_{Acac} = ml. CO_2 acetoacetic acid formed per mg. dry wt. of tissue per hour.
1 With 0.08M acetate.
2 Without acetate.

sis, while hyperthyroidic liver slices show strikingly less acetoacetate formation from acetate (Spirtes *et al.*, 1953) and this condition is also known to lower blood and liver cholesterol (Handler, 1948).

Gurin and Brady (1951) remark that all tissues capable of synthesising cholesterol "in vitro" must have an obligatory mechanism for the production of acetoacetate. This view has been strengthened by our observation that acetoacetate production by kidney is greatly increased in hypothyroidism.

SUMMARY

Free and ester cholesterol values in blood, liver and kidney are increased in hypothyroidic rats (with 0.16% METU in the diet) in comparison to the control pair fed rats maintained for 6–7 weeks.

Q_{O_2} values with acetate as substrate showed a decrease in hypothyroidism in liver and kidney slices while Q_{Acac} values for the formation of acetoacetate showed a rise in such condition.

Possibility of a relation between decreased Q_{O_2} and increased Q_{Acac} and cholesterolegenesis has been discussed.

REFERENCES

Boreld, U. and Hj. Holmgreen: *Endocrinology* **42**: 427. 1948.
Brady, R. O. and S. Gurin: *J. Biol. Chem.* **189**: 371. 1951.
Curran, G. L.: *J. Biol. Chem.*, **200**: 17. 1953.
Edson, N. L.: *Biochem. J.* **29**: 2082. 1935.
Gurin, S. and R. O. Brady: CIBA Foundation Conference on Isotopes in Biochemistry, p. 23 (1951)
Handler, P.: *J. Biol. Chem.* **173**: 295. 1948.
Kennely, B. and L. A. Maynard: *J. Nutrition* **49**: 599. 1952.
Krebs, H. A.: *Advances in Enzymol* **3**: 191. 1943.
Krebs, H. A. and W. A. Jhonson: *Enzymologia* **4**: 148. 1937.
Medes, G., S. Weinhouse and N. F. Floyd: *J. Biol. Chem.* **157**: 751. 1945 a.
Medes, G., S. Weinhouse and N. F. Floyd: *Fed. Proc.* **4**: 98. 1945 b.
Mookerjea, S. and D. P. Sadhu: *Ind. J. Physiol. & Allied Sci.* **8**: 121. 1954.
Mookerjea, S. and D. P. Sadhu: *Ind. J. Physiol. & Allied Sci.*, **9**: 1955 (In Press).
Sobel, A. E. and A. M. Mayer: *J. Biol. Chem.* **157**: 255. 1945.
Spirtes, M. A., G. Medes and S. Weinhouse: *J. Biol. Chem.* **204**: 705 1953.
Srere, P. A., I. L. Chaikoff, S. S. Treitman and L. S. Burstein: *J. Biol. Chem.* **182**: 624. 1950.
Tipton, S. R. and W. L. Nixon: *Endocrinology* **39**: 300. 1946.
Umbreit, W. W., R. H. Burris and J. F. Stauffer: Manometric Techniques, Burgess Minneapolis, Minn., 1945.

THE CONVERSION OF DESOXYCORTICOSTERONE ACETATE TO A PROGESTERONE-LIKE SUBSTANCE[1]

E. A. LAZO-WASEM[2] AND M. X. ZARROW

Department of Biological Sciences, Purdue University, Lafayette, Indiana

IT HAS been known for some time that desoxycorticosterone acetate (DCA) possesses weak progestational activity when administered systemically. However, DCA will not bring about progestational changes when applied topically to the endometrium of the rabbit (McGinty, 1942) nor will it cause a positive reaction in the stromal nuclei of the uterus of the mouse following local application (Hooker and Forbes, 1949). Pfiffner (1942) concluded from an analysis of the structural similarities of the two molecules and from McGinty's data (1942) that desoxycorticosterone is converted in the body to progesterone, probably by removal of the primary hydroxyl group. Since then, evidence has been presented for the *in vivo* conversion of DCA to progesterone in monkeys (Zarrow, Hisaw and Bryans, 1950) but no information is available regarding the locus of this conversion.

MATERIAL AND METHODS

Adult rats of the Sprague-Dawley strain and grey Chinchilla rabbits were used for the studies on the site of conversion of DCA to progestin. The operative procedures were carried out under ether anesthesia or under Nembutal anesthesia supplemented with ether. Adult, ovariectomized mice of the Rockland-Swiss strain were used in all essays of serum progestin.

The determinations for progesterone-like activity were carried out by the method of Hooker and Forbes (1947). The injected horn and the contralateral horn were studied histologically to determine whether or not a positive reaction was due to the systemic effect of desoxycorticosterone. Only those assays were considered positive for progesterone-like activity wherein the injected horn gave a positive reaction while the contralateral horn failed to show stimulation. Since evidence for the identity of the substance measured by the Hooker-Forbes test is lacking, the term progestin or progestogen will be used for the progestational activity measured in these experiments. In keeping with previous studies, however, the activity of the progestin has been standardized against progesterone (Zarrow and Neher, 1953) and will be expressed as μg. equivalents of progesterone.

For the *in vivo* studies, all animals were administered an intramuscular dose of DCA in oil. Blood was obtained by cardiac puncture in rats or from the marginal ear vein in rabbits. The blood was allowed to clot at room temperature, centrifuged and the serum

Received September 20, 1954.

[1] Aided by a grant of the Purdue Research Foundation.

[2] Present address: The Wilson Laboratories, 4221–4223 South Western Avenue Boulevard, Chicago 9, Illinois.

stored in the frozen state until the time of assay. For the *in vitro* studies, fresh or boiled rat tissue slices of various organs were incubated with Krebs-Ringer's phosphate medium with added glucose (Umbriet *et al.*, 1949) under aerobic conditions at 38° C. Following a 6-hour incubation period, aliquot samples of the supernatant fluid were removed and kept frozen until the time of assay. Five mg. of crystalline DCA powder were added to the incubation medium in the experimental flasks.

RESULTS

In vivo studies in the rat and the rabbit

The serum progestin levels of intact or castrated male or castrated female rats and rabbits were observed to vary between 0 and 1 μg. per ml. Following the administration of 5 mg. of DCA to castrated rats, a maximum level of 3.3 μg. of progestin per ml. of serum was observed within 4 hours with a return to preinjection levels by the end of 24 hours. Bilateral adrenalectomy or nephrectomy followed by a similar dose of DCA resulted in maximum serum progestin levels of 2 to 3 μg. per ml. Rats deprived of adrenals and kidneys showed no detectable, circulating progestin following DCA administration (Fig. 1).

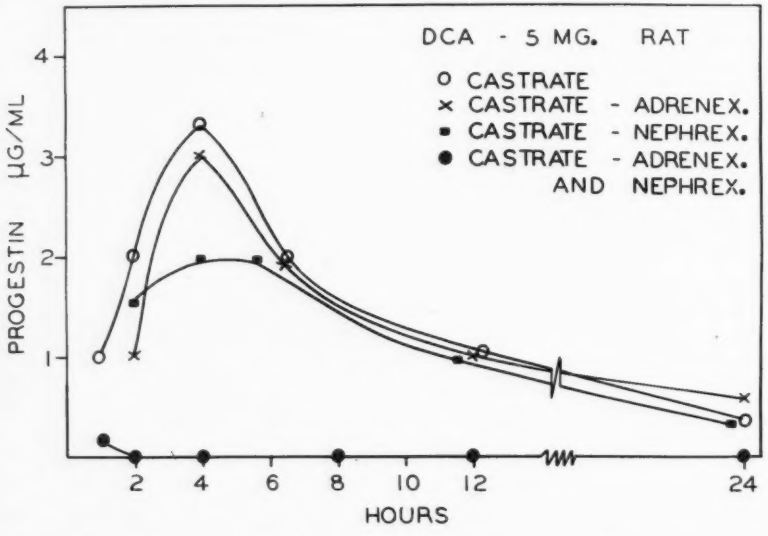


FIG. 1. Serum progestin levels in the rat following treatment with DCA. Each point represents the average of at least two animals.

Following the administration of 40 mg. of DCA to castrated rabbits, a mean serum progestin level of 1.2 and of 4.0 μg. per ml. was obtained at 2 and 4 hours after treatment respectively. A gradual decrease to pre-treatment levels was observed in 36 hours. Serum progestin concentration following treatment with DCA in hepatectomized-castrated rabbits was similar to that observed in normal animals. Nephrectomized rabbits treated

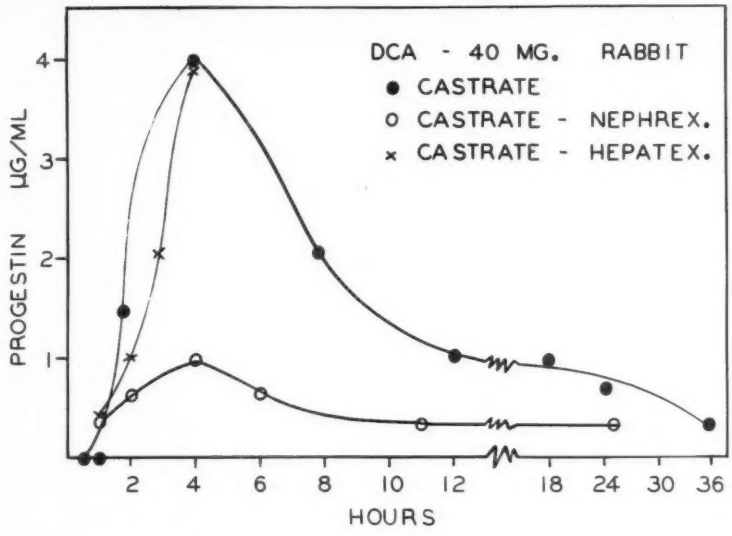


FIG. 2. Serum progestin levels in the rabbit following treatment with DCA. Each point represents the average of at least two animals.

with DCA, however, failed to show a definite increase in circulating progestin above pre-treatment levels (Fig. 2).

In vitro studies with rat tissue slices

Incubation of rat tissue slices with 5 mg. of DCA indicated conversion of DCA to progestin by kidney and adrenal slices. Fresh kidney of intact animals incubated with DCA showed a concentration in the incubation fluid of 6.0 µg. of progestin per ml., while similar kidney slices incubated in the absence of DCA and boiled kidney slices incubated with this steroid yielded only a minimal concentration of 0.3 µg. per ml. The incubation of kidney slices of adrenalectomized rats incubated without DCA showed no detectable progestin. Slices from adrenal glands of intact rats incubated with DCA showed a progestin concentration in the incubation medium of 2.0 µg. per ml., while control adrenal tissue and boiled adrenal slices incubated with DCA resulted in a concentration of only 0.3 µg. per ml. No detectable progestin was observed in the incubation fluid of spleen, liver, muscle, blood or uterus of diestrous rats incubated in the presence of DCA. The incubation medium of uterine slices from pseudo-pregnant rats showed a progestin level of 1 µg. per ml. Since the concentration of progestin was not increased by incubation of uterine slices with DCA, this was taken to indicate a lack of conversion by the uterus (Table 1).

DISCUSSION

The secretion of progestin by the adrenal gland has been suspected since the early experiments of Engelhart (1930) and Callow and Parkes (1936).

TABLE 1. CONVERSION OF DESOXYCORTICOSTERONE ACETATE TO PROGESTIN BY RAT TISSUE SLICES

Tissue	Amount of tissue	Volume of fluid	DCA added	Progestin in incubation medium, μg. per ml.
Fresh Kidney of Intact Rat	500 mg.	2.50 ml.	5.0 mg.	6.0
Fresh Kidney pf Intact Rat	500 mg.	2.50 ml.	None	0.3
Boiled Kidney of Intact Rat	500 mg.	2.50 ml.	5.0 mg.	0.3
Fresh Kidney of Adrenalectomized Rat	500 mg.	2.50 ml.	None	0.0
Fresh Adrenals of Intact Rat	250 mg.	1.25 ml.	2.5 mg.	2.0
Fresh Adrenals of Intact Rat	250 mg.	1.25 ml.	None	0.3
Boiled Adrenals of Intact Rat	250 mg.	1.25 ml.	2.5 mg.	0.3
Fresh Uterus of Pseudopregnant Rat	500 mg.	2.50 ml.	5.0 mg.	1.0
Fresh Uterus of Pseudopregnant Rat	500 mg.	2.50 ml.	None	1.0
Fresh Uterus of Diestrous Rat	500 mg.	2.50 ml.	5.0 mg.	0.0
Fresh Liver of Intact Rat	500 mg.	2.50 ml.	5.0 mg.	0.0
Fresh Spleen of Intact Rat	500 mg.	2.50 ml.	5.0 mg.	0.0
Fresh Muscle of Intact Rat	500 mg.	2.50 ml.	5.0 mg.	0.0
Fresh Blood of Intact Rat	0.5 ml.	2.50 ml.	5.0 mg.	0.0

Further evidence for such a concept was presented by Beall and Reichstein (1938) when they extracted progesterone from the adrenal glands of oxen. The presence of progesterone-like activity in the blood of the male has been shown in the bird (Fraps, Hooker and Forbes, 1949) and in the mammal (Lazo-Wasem *et al.*, 1954). Evidence obtained in the rat would indicate that the adrenal gland may be a source of progestin (Zarrow and Lazo-Wasem, 1953; Lazo-Wasem *et al.*, 1954). Further evidence for the elaboration of progestin by the adrenal gland may be found in experiments on the production of deciduomata in the hypophysectomized-castrated rat treated with adrenocorticotropin (Lyons *et al.*, 1953) and the increased concentration of pregnanediol in the urine of women treated with ACTH (Davis *et al.*, 1953) and men treated with ACTH (Zimmerman and Wojack 1952).

Although the available data indicate that the adrenal gland is a source of progestin, it is not known whether the hormone is secreted specifically or whether it results from a conversion of desoxycorticosterone or a similar steroid. The possibility of DCA conversion to progestin has long been suggested since DCA possesses progesterone-like activity (Courrier, 1940; Gross *et al.*, 1942) only when administered systemically (McGinty, 1942; Hooker and Forbes, 1949). The present results indicate that the conver-

sion of DCA to progestin can occur in the rat and rabbit and that both the kidney and adrenal gland are sites of conversion. Both *in vitro* and *in vivo* experiments confirm this and in general the results indicate that the major site of conversion resides in the kidney.

SUMMARY

1. The conversion of desoxycorticosterone acetate to progestin was studied in two mammalian species.

2. Following the administration of DCA to castrated or castrated-adrenalectomized rats, maximal increases in the serum progestin concentration were observed within 4 hours with a return to normal within 24 hours. Similar results, but of a lower order of magnitude, were observed when castrated-nephrectomized rats were used. No conversion was detected in castrated rats deprived of their kidneys and adrenal glands.

3. Administration of DCA to castrated and castrated-hepatectomized rabbits caused an increase in circulating progestin similar to that obtained in the rat. Removal of the kidneys resulted in a significantly decreased conversion.

4. Incubation of rat tissues with and without the addition of DCA indicated the possibility that the kidneys and the adrenal glands are involved in the conversion of DCA to progestin.

REFERENCES

BEALL, D. AND T. REICHSTEIN: *Nature* **142:** 479. 1938.

CALLOW, R. K. AND A. S. PARKES: *J. Physiol.* **87:** 28P. 1936.

COURRIER, R.: *Press. Med.* **48:** 658. 1940.

DAVIS, M. E., C. E. TEST, C. A. NAVORI, B. HYRSE, R. POTTINGER AND F. DUNKLE: *J. Clin. Endocrinol. & Metab.* **13:** 181. 1953.

ENGELHART, E.: *Klin. Wochenschr.* **9:** 2114. 1930.

FRAPS, R. M., C. W. HOOKER AND T. R. FORBES: *Science* **109:** 493. 1949.

GROSS, G., J. BENOIT, R. KEHL AND R. PARIS: *Compt. rend. Soc. de Biol.* **136:** 749. 1942.

HOOKER, C. W. AND T. R. FORBES: *Endocrinology* **41:** 158. 1947.

HOOKER, C. W. AND T. R. FORBES: *Endocrinology* **45:** 71. 1949.

LAZO-WASEM, E. A., G. M. NEHER, R. L. SHOGER AND M. X. ZARROW: *Endokrinol.* **31:** 166. 1954.

LYONS, W. R., C. H. LI, R. E. JOHNSON AND R. D. COLE: *Proc. Soc. Exp. Biol. and Med.* **84:** 356. 1953.

MCGINTY, D. A. Quoted by Pfiffner: *Advances in Enzymol.* **2:** 325. 1942.

PFIFFNER, J. J.: *Advances in Enzymol.* **2:** 325. 1942.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER: Manometric Techniques and Tissue Metabolism, 2nd. Edition, Burgess Publishing Co., Minneapolis 15, Minn., 1949.

ZARROW, M. X., F. L. HISAW AND F. BRYANS: *Endocrinology* **46:** 403. 1950.

ZARROW, M. X. AND E. A. LAZO-WASEM: Proceedings from the XIX International Physiological Congress, Montreal, Canada, 1953.

ZARROW, M. X. AND H. M. NEHER: *J. Clin. Endocrinol. and Metab.* **13:** 203. 1953.

ZIMMERMANN, B. AND A. J. WOJACK: *J. Clin. Endocrinol. and Metab.* **12:** 972. 1952.

EFFECT OF CORTICOIDS UPON THE DEVELOPMENT AND TOXICITY OF TRANSPLANTABLE CROTON-POUCH TUMORS[1]

HANS SELYE

Institut de Médecine et de Chirurgie expérimentales, Université de Montréal, Montreal, Canada

USING a modification of the "granuloma-pouch" technique—which had originally been designed for the quantitative assessment of inflammation (Selye, 1953a, 1953b)—it has been possible to expose connective tissue to 100% croton oil for periods of many months and thus to induce transplantable malignant neoplasms by treatment with this inflammatory irritant alone (Selye, 1954a). One of the cancers so induced, "croton-pouch tumor No. 2," originated as a typical fibrosarcoma in the croton-oil treated animal, but after a few animal-passages, it developed a tendency to form cartilaginous and bony islets so that it now bears the characteristics of a fibro-chondro-osteosarcoma. It takes in 100% of the cases when transplanted as a tissue-suspension, intramuscularly or into a subcutaneous air-sac (such as is used for the production of granuloma-pouches), into Sprague-Dawley rats, but Wistar rats appear to be immune to it. Grown in the granuloma-pouch it produces large quantities of a viscous exudate which is extremely toxic and renders the blood incoagulable, when injected intravenously.

It had also been demonstrated that the inhibition of various transplantable neoplasms of the mouse (EO771, 15091A) or rat (Walker No. 256) by cortisone or cortisol,[2] can be in turn abolished and the normal tumor-growth-rate restored if the animals are simultaneously treated with somatotrophic hormone (STH) (Selye, 1954b, 1954c). In many respects, prophologistic corticoids, such as desoxycorticosterone acetate (DCA), antagonize the actions of antiphlogistic corticoids, such as cortisone or cortisol, just as effectively as STH. It was considered of interest, therefore, to determine whether that would also be the case as regards the effect of cortisol upon a transplantable tumor.

Received for publication September 28, 1954.

[1] These investigations were performed with the aid of a grant from the National Cancer Institute of Canada.

[2] In agreement with the suggestion made by Shoppee (1953), the term cortisol is used here in preference to "hydrocortisone" since it eliminates confusion with 4,5-dihydrocortisone and obviates the possible implication that "hydrocortisone" is to be regarded primarily as a mere derivative of cortisone.

Interestingly, the various characteristic actions (catabolic, lympholytic and antiphlogistic) of cortisol are much more evidently antagonized by DCA in adrenalectomized than in intact animals (Selye, 1954d). Apparently, the living adrenal acts as a buffer and exerts some neutralizing effect through its own hormones which obscures the typical actions of injected corticoids.

In the experiments to be reported here, we examined the effect of DCA and cortisol, singly and in combination, as regards their ability to influence the growth and the systemic toxicity of croton-pouch tumor No. 2 transplants, both in intact and in adrenalectomized rats.

EXPERIMENTAL PROCEDURE

Seventy Sprague-Dawley rats, having an average body weight of 133 gm. (range 122–147 gm.), were subdivided into seven equal groups and treated as outlined in Table 1.

Bilateral adrenalectomies were performed in Groups V–VII, through the lumbar route and treatment with the steroids was begun on the same day. Microcrystal suspensions of cortisol acetate[3] (250 μg./day) and DCA[3] (1 mg./day) were administered it 0.2 ml. of water, both steroids being injected subcutaneously in the ventral region.

TABLE 1. EFFECT OF CORTICOIDS UPON THE GROWTH OF TRANSPLANTED "CROTON-POUCH TUMOR NO. 2" AND THE BODY'S REACTION TO THIS NEOPLASM

Group	Treatment	Adrenal wt. in mg.	Exudate in ml.	Tumor in gm.	Thymus in mg.	Spleen in mg.	Body wt. gain in gm.	Death in %
I	Intact No treatment	37.4±1.8	45±4.4	25.4±3.7	148±20	651±79	+14 ±2	0
II	Intact Cortisol	40.7±1.6	34±5.9	15.6±1.3	72±20	399±39	+ 7.6±1.8	0
III	Intact DCA	45.2±1.3	58±3.8	25.1±2.4	147±35	624±81	+17 ±3.1	0
IV	Intact Cortisol+DCA	41.3±1.7	38±6.7	19 ±1.5	61±7	477±42	+19 ±3.4	0
V*	Adrenalectomized Cortisol	—	Trace	5.4±2.5	55±8.5	388±82	−29 ±3.2	70
VI	Adrenalectomized DCA	—	36±3.5	16 ±1.6	403±22	942±32	+40 ±5.1	0
VII	Adrenalectomized Cortisol+DCA	—	37±4	16 ±1.5	89±17	519±42	+17 ±2.1	0

* All values in this column represent averages of animals which survived until the termination of the experiment.

In the rats of all groups neoplastic granuloma-pouches were prepared two days later, beneath the shaved skin of the back in the usual manner (Selye, 1953a, 1954b). Under sterile conditions and in ether anesthesia 25 ml. of air was first injected, this being immediately followed by the introduction of 1 ml. of tumor suspension. The latter was prepared by crushing the tissue of a two-week-old granuloma-pouch tumor No. 2, in a glass homogenizer, after adding twice its volume of physiologic saline.

[3] The author wishes to thank Merck and Co., Inc. for a generous supply of cortisol (Hydrocortone Acetate). He is also grateful to the Schering Corporation for furnishing the desoxycorticosterone acetate.

The rats were maintained exclusively on "Purina Fox Chow" and tap water. The growth of the tumors and the formation of exudate were followed daily by transillumination with an electric flashlight and all surviving rats were killed 12 days after transplantation of the sarcoma tissue.

Immediately after autopsy, the exudate in the neoplastic granuloma-pouches was measured, by aspiration into a graduated syringe. At the same time the tumor tissue, the adrenals, thymuses and spleens of all animals were dissected and fixed in Susa solution for subsequent weighing and histologic study.

EXPERIMENTAL OBSERVATIONS

Our principal observations are summarized in Table 1 so that only the salient features will require special comment.

It should be said at the outset that preliminary experiments showed the impossibility of obtaining a survival of more than a few days in untreated, adrenalectomized, tumor-bearers, because they cannot stand the stress of these highly toxic neoplasms. It is for this reason that we have only three groups in the adrenalectomized series.

The *adrenals* of the intact-untreated rats (Group I) are unusually small for animals of this weight-range (norm: 53.6 ± 3.6 in our colony), especially in view of the large masses of tumor tissue which manifestly caused stress. The heaviest adrenals are those of the DCA-treated group, but in view of the comparatively high standard error, the significance of these apparent differences are subject to doubt.

Both the *exudate*-forming ability and the actual growth of the transplanted *croton-pouch tumor* are significantly inhibited by cortisol, but only in adrenalectomized animals (Group V). Under our experimental conditions, DCA in itself does not affect these neoplasms in a very clearcut manner, but it virtually abolishes the above mentioned inhibitory actions of cortisol (Group VII), as illustrated also in Figures 1 and 2.[4]

The involution of the *lymphatic organs* was apparently so severe—under the combined effect of cortisol and the stress of the transplanted neoplasm—that here the usual antilympholytic (anticortisol) effect of DCA (Selye, 1954d) could not manifest itself clearly. It was absent as regards the thymus and doubtful as regards the spleen of intact animals (Group IV). After adrenalectomy, there was a slight antagonistic action in this respect also but, when comparing the weights of the thymuses ($P = 0.2$) and spleens ($P = 0.2$) in Groups V and VII, this did not prove to be statistically significant. A marked loss of *body-weight* and a high *mortality* were

[4] Of course, in the absence of a comparable untreated-adrenalectomized group, the possibility had to be considered that the dose of cortisol given was too small to have an optimal "normalizing" effect. If this were so, additional treatment with DCA could have acted merely by improving the well-being of the animals through the supply of more corticoid activity, without there being any actual antagonism between the two hormones. However, numerous control experiments, performed under similar conditions, with ascending doses of cortisol (up to 2.5 mg./day), showed that the inhibitory effect of cortisol merely becomes increasingly more obvious (Selye, 1954e).

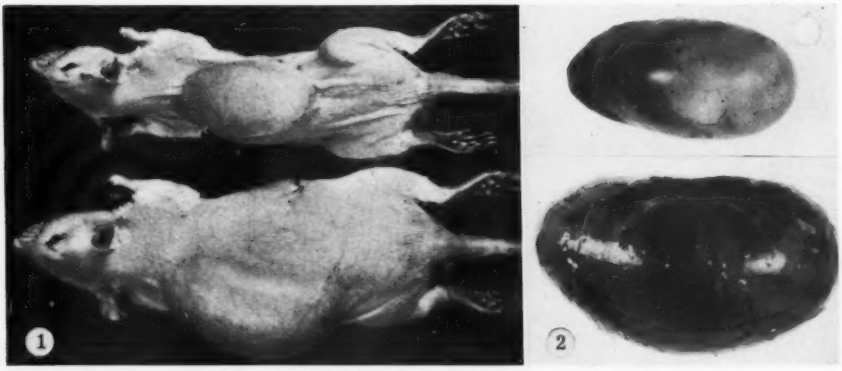


FIG. 1. Macroscopic aspect of adrenalectomized rats treated with cortisol alone (top) or cortisol and DCA (bottom). The neoplastic granuloma-pouch containing the transplanted "croton-pouch tumor No. 2" is very small in the rat maintained on cortisone alone and the animal shows signs of growth-inhibition and cachexia. The rat receiving the same amount of cortisol together with DCA is essentially of normal size and bears a much larger pouch.

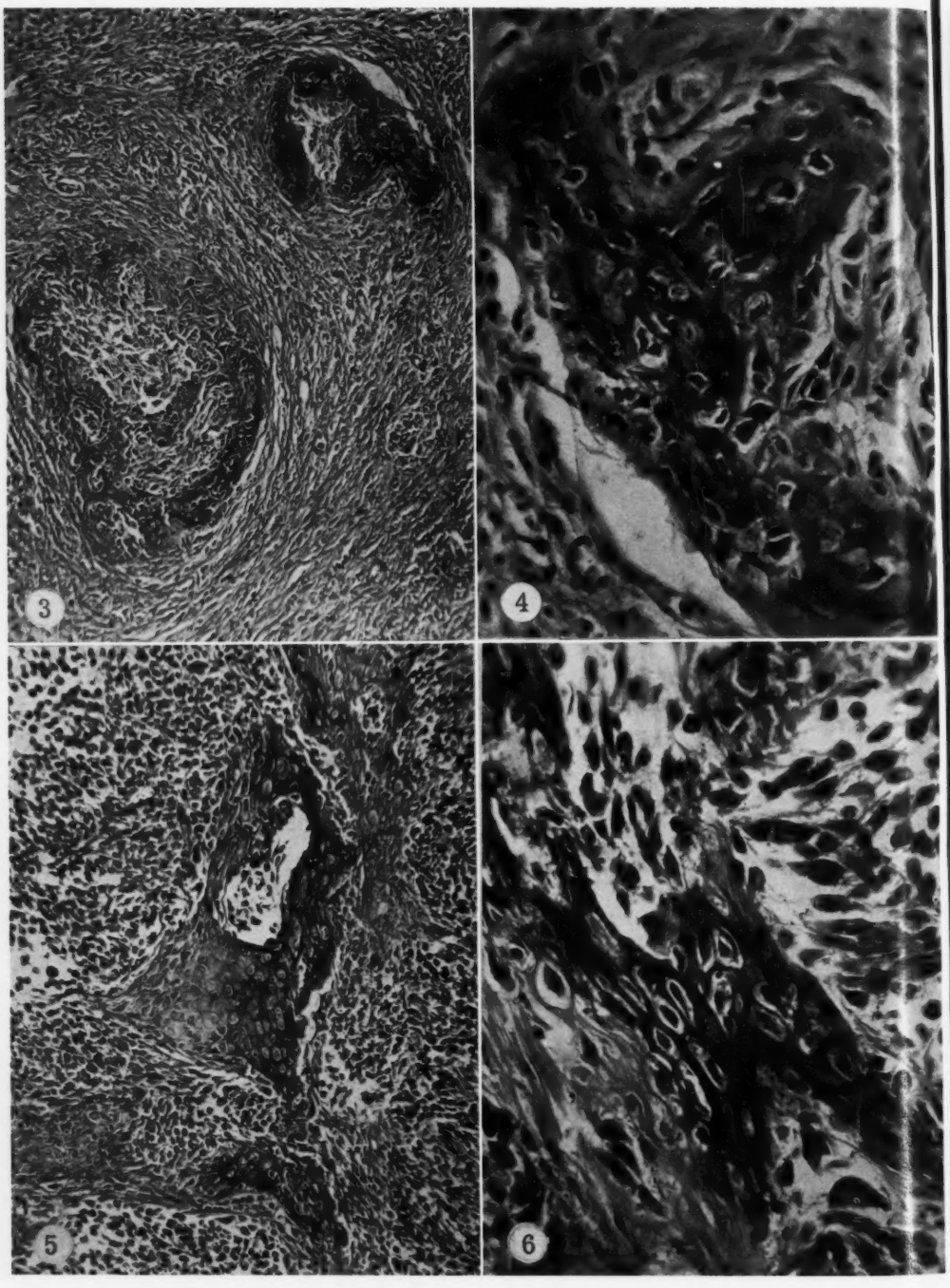
FIG. 2. Dissected neoplastic granuloma-pouches of the rats shown in Fig. 1. Note that the animals treated with cortisol secreted no exudate into the air-sac, while the rats simultaneously given DCA produced a large amount of foamy exudate which can clearly be seen through the transparent "roof" of the pouch.

induced by cortisol in these tumor-bearing animals only after adrenalectomy (Group V). Conversely, adrenalectomized rats, maintained exclusively on DCA, support their tumors without mortality and are even capable of considerable growth (Group VI). Here, the cortisol-neutralizing action of DCA is again clearly evident as regards both the body-weight and the mortality (Group VII).

The slowly growing and perhaps involuting tumors, in the few surviving rats of Group V, have been subjected to a careful *histologic study*, which revealed that the actual size and proliferation of the sarcoma cells were greatly diminished by cortisol, but again only in the absence of adrenal tissue. Thus, in Group V, the tumors became relatively acellular and fibrous and the osteosarcomatous areas assumed an appearance more similar to that of normal bone trabeculae (Figs. 3 and 4). However, simultaneous treatment with DCA restored the original, highly malignant aspect of these growths so that they proved to be indistinguishable from the tumors of the intact controls. The cells were large, atypical forms and mitoses were frequent, while fibrous areas were scarce. (Cf. figs. 5 and 6.)

DISCUSSION

It is especially significant that, in all these respects, the actions of cortisol are inhibited by the presence of a living adrenal (Group II) approximately in the same manner as—after adrenalectomy—by the injection of


3
4
5
6

DCA (Group V). It will be recalled that, in the first studies concerning the ability of DCA to exert prophlogistic effects (myocarditis, periarteritis nodosa, arthritis), it was found necessary to remove the adrenals in order to obtain maximal overdosage effects (Selye *et al.*, 1944). Subsequently, several investigators reported observations, both in animals and in Addisonian patients, indicating that sensitivity to injected corticoids is greatly enchanced by removing the adrenals. (For review of pertient literature cf. Selye, 1954d, 1950, 1951, Selye and Horava, 1952, 1953.)

In evaluating the experiments reported in this paper it is of special interest that according to Ingle (1952) various metabolic actions of cortisone overdosage are also enhanced by adrenalectomy. The impression was gained in comparing groups of intact and adrenalectomized cortisone-treated rats, that some of the organ-changes produced by cortisone may have been accentuated in the absence of the adrenals. However, Ingle has carefully concluded that since, under the experimental conditions which he used, there existed "some difficulty in appraising these changes quantitatively and objectively, no claim is made that we have proven a true difference in the incidence and extent of pathology between these two groups."

Ingle (1952) also raised the interesting question "Does it (the adrenal) have the capacity to inactivate some exogenous cortisone or does it continue to secrete substances which either balance or inhibit the effects of cortisone?" This point is of the greatest interest in connection with our theory of stress and the diseases of adaptation, which postulates the importance of an antagonistic balance between pro- and antiphlogistic corticoids in health and disease.

Although adrenal-inactivation of corticoids may also occur, the observations reported in this paper clearly indicate that we need not postulate it to explain the observed facts, since even after adrenalectomy the most varied actions of cortisol are antagonized by simultaneous DCA treatment.

SUMMARY

The growth and systemic toxicity of transplanted "croton-pouch tumor No. 2" (a highly malignant, fibro-chondro-osteosarcoma) was examined in

---

←

FIG. 3. Low magnification of bony spicules and the fibrous, comparatively acellular sarcoma tissue in the cortisol treated rat shown in Figure 1.

FIG. 4. An area from the tumor shown in Figure 3, under high magnification. Here again, the comparatively shrunken cells in and around the bone-spicule are clearly visible.

FIG. 5. Region from the cortisol plus DCA treated rat (shown in Fig. 1) under the same magnification as Figure 3. Note that the cells are much larger and fibrous tissue is almost missing around the partly cartilaginous and partly bony spicules.

FIG. 6. Region from the tumor shown in Figure 5, under higher magnification.

intact and in adrenalectomized rats treated with cortisol, DCA or both these steroids.

Using the "neoplastic granuloma-pouch technique"—in which tumor-tissue suspensions are introduced into regular, symmetrical subcutaneous air-sacs—it was found that cortisol greatly inhibits both the growth and the exudate-forming ability of croton-pouch sarcomas, but only in the absence of adrenal tissue.

The anticortisol action of the living adrenal could be duplicated, after adrenalectomy, but simultaneous treatment with DCA.

These findings—as well as the effect of cortisol upon the body-weight and death-rate of these tumor-bearing rats—suggest that DCA imitates the cortisol-antagonizing effect of intact adrenal tissue in many respects.

Acknowledgments

The author is indebted to Doctor J. Ventura for statistical calculations and to Mr. Kai Nielsen for the microphotographs.

REFERENCES

INGLE, D. J.: *Proc. Soc. Exper. Biol. & Med.* **79**: 184. 1952.

SELYE, H.: *Stress, The Physiology and Pathology of Exposure to Stress*, Acta Inc., Montreal, 1950.

SELYE, H.: *First Annual Report on Stress*, Acta Inc., Montreal, 1951.

SELYE, H.: *J.A.M.A.* **152**: 1207. 1953a.

SELYE, H.: *Proc. Soc. Exper. Biol. & Med.* **82**: 328, 1953b.

SELYE, H.: *J. Nat. Cancer Inst.*, 1954a (in press).

SELYE, H.: *Proc. First Canad. Cancer Res. Conf.*, Honey Harbour, Ont., 1954b. (Acad. Press, Inc., New York, 1955)

SELYE, H.: *Ztschr. Krebsforsch.*, 1954c (in press).

SELYE, H.: In: *Fourth Annual Report on Stress*, by H. Selye and G. Heuser, Acta Inc., Montreal, 1954d.

SELYE, H.: Unpublished, 1954e.

SELYE, H. AND H. HORAVA: *Second Annual Report on Stress*, Acta Inc., Montreal, 1952.

SELYE, H. AND H. HORAVA: *Third Annual Report on Stress*, Acta Inc., Montreal, 1953.

SELYE, H., O. SYLVESTER, C. E. HALL, AND C. P. LEBLOND: *J.A.M.A.* **124**: 201. 1944.

SHOPPEE, C. W.: In: *Annual Rev. Biochem.*, **22**: 261. 1953.

IN VITRO BIOASSAY OF CORTICOTROPIN: MODIFICATION AND STATISTICAL TREATMENT

MURRAY SAFFRAN AND ANDREW V. SCHALLY

Allan Memorial Institute of Psychiatry, McGill University, Montreal, Quebec

WE HAVE previously (Saffran and Bayliss, 1953) described an *in vitro* system that can be used for estimating the potency of preparations of corticotropin, but the experimental design described then did not permit statistical evaluation of the data. The procedure has been modified to allow the use of standard computational methods for the calculation of the potency, and its limits, of an unknown preparation relative to a standard. (Pugsley, 1946; Bliss, 1951).

The modified method is relatively rapid and convenient to carry out and its precision compares favorably with other bioassay methods.

METHOD

Eight young adult rats (125 to 200 gm) of the Sprague-Dawley strain are used in each assay[1]. The rats are anesthetized by the intraperitoneal injection of 5 mg. of nembutal per 100 gm. of rat[2]. The adrenals are removed from a rat and are carefully freed of adhering fat with fine scissors, taking care that the adrenal is not damaged. As soon as a pair of adrenals is cleaned and weighed on a micro torsion balance[3] the glands are placed into a petri dish containing some incubation medium[4] and are carefully cut into quarters with fine sharp scissors. The quartering should be as even as possible; eight almost-equal quarters can be cut from a pair of adrenals after a little practice. The quarters are then placed into eight numbered sectors of a filter paper circle moistened with medium and kept in a petri dish in a humidor[5]. The adrenals of the eight rats are thus cleaned, quart-

Received October 22, 1954.

[1] No special study has been made of the influence of the age, sex or strain of rat on the sensitivity or the precision of the assay. The number of rats per assay is not critical, but enough tissue must be used in each flask to ensure the production of a measureable amount of hormones. The only stipulation is that the adrenals of each rat must be divided as equally as possible among eight flasks. As few as 4 animals have been used successfully.

[2] We usually anesthetize the rats in the animal room to avoid disturbing them during transport to the laboratory.

[3] This weighing step is precautionary and merely serves to detect any large change in weight of the tissue during the next few steps. The weighing of the intact glands may be omitted after experience proves that the weight changes are negligible.

[4] The medium is Krebs-Ringer-Bicarbonate Solution (Umbreit, Burris and Stauffer, 1949) containing 200 mg. % glucose. Bicarbonate-Buffered medium is preferred to avoid the danger of precipitating the calcium by phosphate (Birmingham, Elliott and Valere, 1953).

[5] The humidor may consist of a covered saucepan entirely lined with filter paper which is kept moist by a layer of water on the bottom.

tered and distributed into eight lots in the humidor, each sector containing a quarter of an adrenal from each of the eight rats.

The adrenal quarters in each sector are weighed on a micro torsion balance and are placed into Warburg flasks containing 1.5 ml. of medium.

The flasks are mounted on Warburg manometers, are well flushed with a mixture of 5% CO_2-95% O_2 and are shaken in the bath at 38° for 1 hour. During this preincubation period[6] the samples of corticotropin are weighed or measured and are diluted with medium so that 0.1 ml. contains 9 mU., for the high doses, and 3 mU. for the low doses[7].

At the end of the preincubation period the flasks are removed from the bath. The medium is sucked out as completely as possible and is discarded. 1.4 ml. of fresh medium

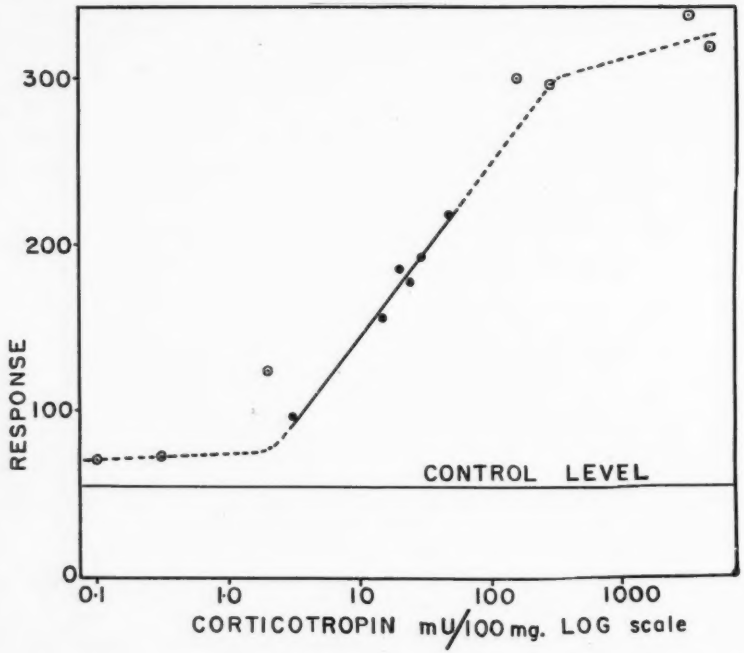


FIG. 1. Dose-response curve. The solid line was calculated by the method of least squares from the solid points. The dashed portions of the line were drawn by inspection through the open points. The control level represents the amount of corticoid produced by adrenals incubated without added corticotropin. All the points were obtained with tissue from the same group of 16 animals divided among the flasks. The response is calculated as described in the text.

Over the solid portion of the curve, $b = 104.54$ and $s = 8.56$, with $\lambda = 0.082$.

---

[6] The preincubation period may be varied from ½ to 1½ hours. During this period a substance that interferes with the ultraviolet measurements is washed out of the tissue (Elliott, Birmingham, Schally and Schonbaum, 1954).

[7] The adrenal response to corticotropin is approximately linearly related to the log dose over the range 3 to 300 mU. per 100 mg. tissue (Fig. 1). However, the linear range varies in different groups of rats. We have found that 10 and 30 mU. per 100 mg. are safe dosage levels with our animals.

is added to each flask. The flasks then receive approximately 0.1 ml. of the appropriate corticotropin solutions[8]. Two of the flasks are given the low dose of standard (S_1); two, the high dose of standard (S_2); the two other pairs receive the low and high doses of the unknown (U_1 and U_2). The solutions of corticotropin are assigned to the flasks at random.

The flasks are again mounted on the manometers, flushed well with the gas mixture, and are replaced into the bath for a two-hour incubation at 38°.

During the incubation period, eight glass-stoppered 2 ml. volumetric tubes are prepared with 1.0 ml. of purified methylene chloride in each.[9]

At the end of the incubation period the flasks are removed from the bath. One ml. aliquots of the medium are transferred from each flask into the 2 ml. tubes. Then the tubes are well stoppered and are shaken. The contents generally emulsify. The stoppered tubes are centrifuged at about 2000 RPM to separate the two phases. It may be necessary to invert the tubes and centrifuge again to ensure a clean separation. A part of the lower phase is transferred with a long needle and syringe to a quartz micro cuvette and readings are taken at 230, 240, 250, 255, and 260 mμ in a Beckman model DU ultraviolet spectrophotometer. The readings at the shortest wavelengths are facilitated by the photomultiplier attachment available for the instrument. The blank cuvette contains methylene chloride which had been shaken with the incubation medium. The lamp housing of the spectrophotometer must be cooled to avoid evaporation of the solvent. During the readings the tubes awaiting attention must be kept stoppered.

The response at each dose is estimated[10] by $\frac{\text{O.D.}_{240} - \text{O.D.}_{255}}{\text{mg. adrenal}} \times 10$.

The calculation of the relative potency of the unknown is carried out by the factorial analysis method of Bliss and Marks (1939), as outlined by Pugsley (1946) and Bliss (1951).

The steps in carrying out the assay[11] are summarized in Table 1.

In our previous work the corticoids were extracted from the incubation medium with chloroform, resulting in a relatively high and variable absorption of blank extractions in the ultraviolet (Elliott *et al.*, 1954). The substitution of methylene chloride for chloroform practically eliminated the interference (Fig. 2) so that the absorption at 240 mμ may be used directly to estimate the corticoids. However, a slight turbidity sometimes develops in methylene chloride extracts and the reading at 240 mμ must be corrected for the turbidity by subtracting the absorption at a higher wave length. The lowest variance, and hence the lowest λ, was obtained by using the absorption at 255 mμ for the correction, probably because

---

[8] The volume of the corticotropin solutions should be adjusted so that the volume/adrenal weight ratio is equal in all flasks.

[9] The methylene chloride is washed with an equal volume of water, dried with anhydrous sodium sulfate and decolorized with charcoal. The solvent is kept over sodium hydroxide flakes for about 24 hours and is then distilled; the fraction boiling between 40° and 41° is collected. Because of the volatility and toxicity of the solvent, the manipulations are carried out in a hood and the solvent is dispensed from an ungreased buret.

[10] The readings are taken at the other wave lengths so that the purity of the substances extracted by the methylene chloride can be judged from the curve (Fig. 2). When there is no peak at about 240 mμ there are interfering substances present and the measurement must be discarded. This occurs only rarely.

[11] Two complete assays can easily be carried out simultaneously.

TABLE 1. OUTLINE OF METHOD AND SAMPLE PROTOCOL

OUTLINE

Time	Operation
9:35	Injection of nembutal into rats.
	Pipetting of 1.5 ml. medium into flasks.
9:50	Removal of adrenals from rats; cleaning and quartering of glands.
10:22	Weighing of adrenals quarters and distribution among the flasks.
10:35	Mounting of flasks on manometers; flushing with gas mixture.
10:49–11:49	Preincubation period.
	Preparation of corticotropin solutions.
11:50	Removal of flasks from bath.
	Removal of preincubation medium.
	Addition of 1.4 ml. new medium to flasks.
	Addition of corticotropin to flasks.
12:13	Mounting of flasks on manometers; flushing with gas mixture.
12:25– 2:25	Incubation period.
2:10	Preparation of extraction tubes with 1.0 ml. methylene chloride.
2:25	Removal of flasks from the bath.
2:30	Transfer of 1 ml. aliquots of medium from the flasks to the extraction tubes; shaking of tubes.
2:50	Centrifugation of the tubes.
	Turn on spectrophotometer.
3:15	Reading of samples.
3:50	Calculation of the results.

SAMPLE PROTOCOL

S_1 3 mU. per 0.1 ml. U_1 28.7 μg. per 0.1 ml.
S_2 9 mU. per 0.1 ml. U_2 86.0 μg. per 0.1 ml.

Flask #	1	2	3	4	5	6	7	8
Adrenal weight, mg.	34.7	33.8	30.0	33.5	33.8	32.6	30.0	33.0
Sample	U_1	U_2	S_1	S_2	S_1	U_1	S_2	U_2
Sample volume, μl.	115	115	100	110	115	110	100	110
Medium volume, ml.	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Optical Densities × 1000								
230 mμ	510	660	370	590	460	530	480	610
240	650	880	472	800	590	680	630	820
250	440	605	308	525	390	450	420	560
255	260	360	178	300	220	260	240	320
260	120	165	75	125	90	110	92	140
240–255	390	520	294	500	370	420	390	500
240–255								
Response = ——— × 10	112	153	98	149	109	128	130	151
Adrenal weight								

Slope, b = 71.3

Root mean square for experimental error, s = 9.59.

Index of precision, $\lambda = s/b = 0.134$.

Log potency ratio M = 0.203. Antilog M = 1.595.

Standard error of $M = s_M = 0.1014$.

Percent error = 100 (antilog $s_M - 1$) = 26%.

Limits of error at $p = 0.05 = 0.203 - (2.776 \times 0.1014)$ and $0.203 + (2.776 \times 0.1014) = \bar{1}.923$ and 0.483. Antilogs = 0.84 and 3.04.

Therefore, 1 mg. contains $\frac{9 \times 1.595}{86} \times 1000 = 167$ mU. of corticotropin activity, with limits of 88 and 318.

the instrument error at the values at 255 mμ is less than the error at the lower readings at higher wavelengths.

The reasons for considering the substances absorbing at 240 mμ to be corticoids have been given previously (Saffran, Grad and Bayliss, 1952). Chromatographic evidence for their identity has been presented recently by Elliott and Schally (1955).

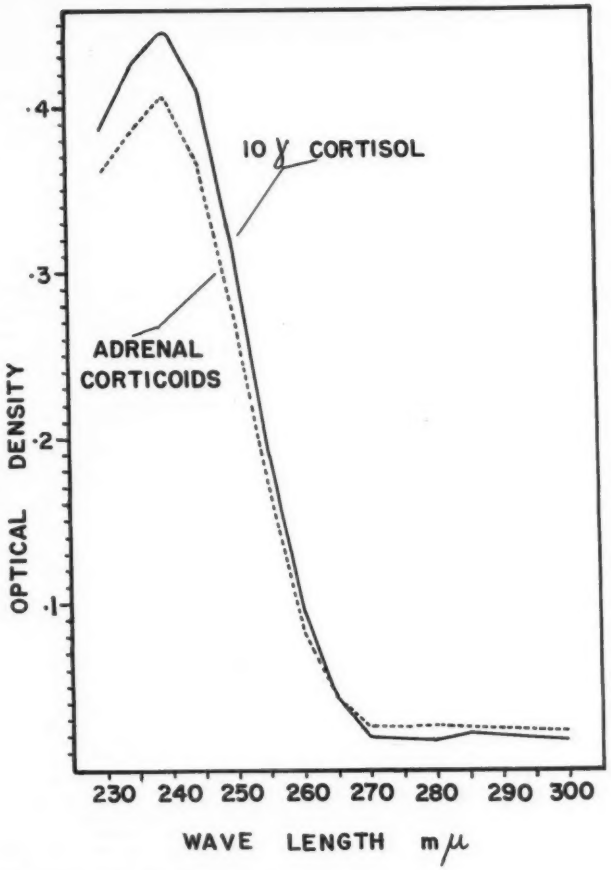


FIG. 2. A comparison of the absorption spectra of cortisol (compound F) in methylene chloride and of a methylene chloride extract of the incubation medium of rat adrenals. Readings were taken at intervals of 5 mμ.

RESULTS AND DISCUSSIONS

Table 2 lists the results of the assay of several pituitary preparations for corticotropin activity by the *in vitro* method.

Agreement with the ascorbic acid method. The claimed potencies of the corticotropin preparations in Table 2 are based on the adrenal ascorbic acid depletion assay of Sayers, Sayers and Woodbury (1948). The results by the two methods agree fairly well, with the biggest discrepancy in the case of the most potent preparation tested, where the *in vitro* assay yielded a somewhat higher potency. Only further comparative assays can reveal whether the two methods will agree when material of various methods of manufacture and various degrees of purity are analyzed.

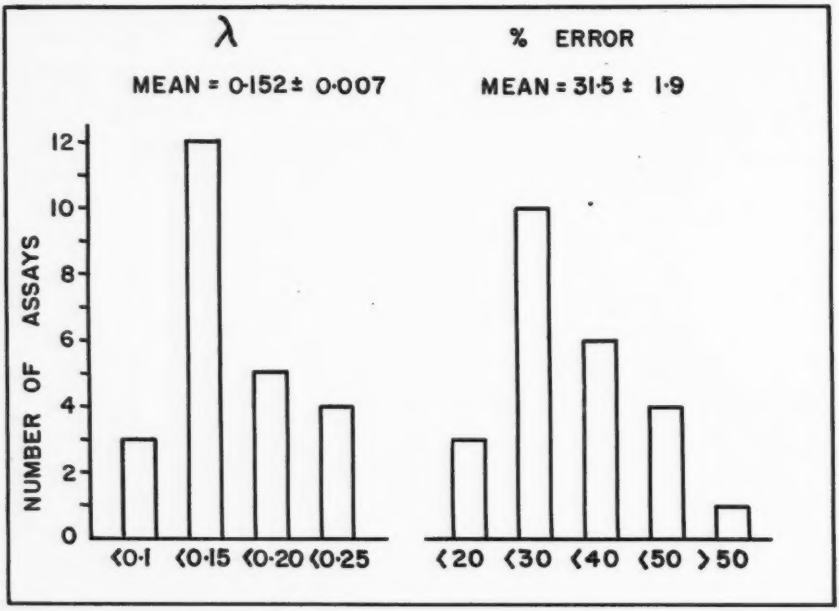


FIG. 3. The distribution of λ and per cent error in 24 assays.

Specificity. Table 2 demonstrates that the *in vitro* assay is relatively specific for corticotropin, since only the corticotropin preparations showed appreciable activity. Hofmann and Davison (1954) have claimed that acetoacetate increases the formation of corticoids by rat adrenals *in vitro*,

TABLE 2. THE ASSAY OF PITUITARY PREPARATIONS FOR CORTICOTROPIN ACTIVITY

Preparation	Source	Claimed potency, Units/mg.	Assayed potency, Units/mg.	λ
Corticotropin	Armour	2.22	1.82 (0.65–4.78)	0.22
	Nordic	11.6 (8.6–14.3)	17.7 (13.3–23.6) 16.5 (9.6–28.6)	0.12 0.21
	Connaught	1.5 (0.9–2.5)	1.42* (1.18–1.70)	0.15
Somatotropin	Horner (a)		0.04 (0.02–0.06) 0.04 (0.02–0.08)	0.13 0.14
	Horner (b)		0.05 (0.03–0.19)	0.23
Thyrotropin	Armour		0.03 (0.01–0.07)	0.08
Melanophore-expanding	Stehle (a)		0.13 (0.07–0.25)	0.13
	Stehle (b)		0.05 (0.02–0.13) 0.06 (0.03–0.12)	0.20 0.15

* Means of 8 determinations. See Table 3.
The potencies are accompanied by their 5% limits, where available.

TABLE 3. REPEATED ASSAYS OF CONNAUGHT CORTICOTROPIN LOT 8-3 AGAINST THE PROVISIONAL U.S.P. STANDARD PREPARATION. STATISTICAL TREATMENT ACCORDING TO BLISS (1951), PAGE 576 *et seq.*

Assay number	M	s_M	Potency U./mg.	λ	% error
1	−0.010	0.087	1.76	0.12	22
2	0.013	0.084	1.85	0.12	21
3	−0.109	0.092	1.40	0.13	24
4	−0.328	0.090	0.84	0.11	23
5	−0.076	0.142	1.51	0.19	38
6	−0.215	0.095	1.10	0.13	24
7	−0.015	0.159	1.74	0.22	44
8	−0.008	0.133	1.77	0.19	36
Unweighted mean	−0.0968	0.110	1.50	0.15	29
Semi-weighted mean	−0.105	0.039	1.42		9

but they failed to find a stimulation by levels of corticotropin which are used in this assay. Birmingham, in our laboratory, has not been able to detect a stimulation by acetoacetate under the conditions of the assay.

Precision. The mean index of precision (λ) of 24 assays is 0.15 ± 0.007, with a distribution that is illustrated in Figure 3. This value of λ is usually considered to be satisfactory in the bioassay of hormones (cf. Sayers *et al.*, 1948).

Reproducibility. Repeated assays on the same preparations yield relatively consistent values (Tables 2 and 3). Table 3 lists eight assays on a sample of corticotropin prepared by the Connaught Laboratories and also demonstrates the result of pooling replicate assays by the method of Bliss (1951); the error computed by Bliss' method is much lower than the error obtained from the unweighted mean.

Error. The mean percent error of 24 assays is $31.5\% \pm 1.94$, with the distribution illustrated in Figure 3. The following sources of error are present:

1. Unequal amounts of reactive tissue in the left and right adrenals.
2. Variable amount of medulla, or other non-responsive adrenal tissue, in the quarters. Unequal responses of the quarters for other reasons.
3. Errors in weighing the tissue.
4. Errors in measuring out the corticotropin.
5. Errors in the extraction and measurement of the corticoids.
6. Errors of unknown origin.

Sources of error 1 and 2 are difficult to control; the only possibility for improvement lies in increasing the accuracy of quartering the glands. The error in weighing the adrenal quarters may be considerable, since the glands must be kept moist. However, speedy handling under standard conditions minimizes this source of error. The error introduced in the measurement of the samples is partly dependent on the variable amount of active tissue present in each flask. This error can be minimized by adjust-

TABLE 4. REPLICATE EXTRACTIONS OF THE SAME INCUBATION MEDIUM

$\frac{OD_{240}-OD_{255}}{\text{mg. tissue}}\times 10$	
78.0	81.0
77.5	82.5
76.0	78.7
84.2	78.0

Mean ± standard deviation = 79.5 ± 2.82.

ing the amount of corticotropin to the weight of tissue in each flask. The extraction and measurement of the corticoids is a relatively small source of error. Table 4 lists 8 replicate extractions of a single sample. A single extraction with an equal volume of methylene chloride recovers more than 95% of the corticoids added to the aqueous medium.

The error and the precision of a bioassay are both affected by the number of test objects in the assay. In the present design only 2 flasks are assigned to each dose of each preparation. It is possible to divide the adrenals into eighths and then assign 4 flasks to each dose in a 16-flask assay. Statistically, the error should decrease, but in actual practice the error is not changed consistently because the difficulty in obtaining equal adrenal eighths counterbalances the advantage obtained by the increased number of flasks per dose. However, we have not performed a large number of 16-flask as-

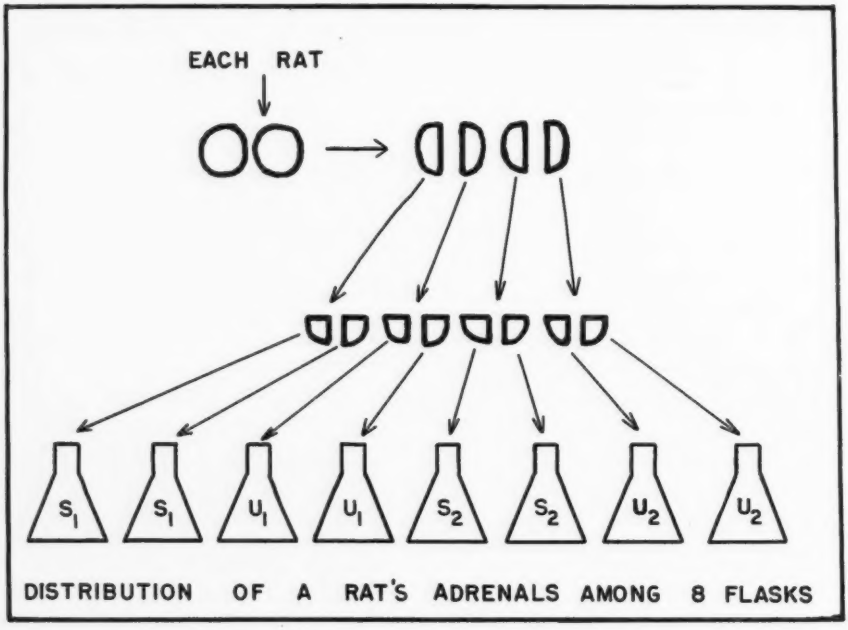


FIG. 4. Distribution of the adrenal tissue of each rat among eight flasks.

says; perhaps the pretreatment of rats with corticotropin for some time will result in adrenals large enough to be divided easily into eighths.

The relatively satisfactory precision of this method with so few test objects can be accounted for by the common origin of the reacting tissue in all flasks (Fig. 4). In most bioassays the test objects are single animals with different sensitivities, in spite of inbreeding and the use of littermates. To assess the degree of improvement resulting from the common origin of the tissue, assays were set up in two ways, with Connaught corticotropin as both standard and unknown: (1) The adrenals of each rat were divided among 8 flasks as usual, and (2) each flask received the quartered adrenals of a different rat. The results (Table 5) demonstrate the efficacy of the distribution of the tissue from each rat among all the flasks, in decreasing the error and improving the precision.

TABLE 5. ASSAY OF CONNAUGHT CORTICOTROPIN LOT 8-3 AGAINST ITSELF. THE EFFECT OF THE DISTRIBUTION OF THE ADRENAL TISSUE AMONG THE FLASKS ON THE PRECISION AND THE ERROR OF THE ASSAY

Standard / Unknown added	Standard / Unknown found	5% limits	λ	% error*
(1) Adrenals from each rat divided among 8 flasks				
1.0	1.04	0.64–1.71	0.11	14
1.0	0.86	0.47–2.94	0.20	39
1.25	1.16	0.99–1.52	0.04	7
1.67	1.34	0.86–2.05	0.09	14
(2) Each flask received adrenals from a different rat				
1.0	1.08	0.045–25.9	0.70	214

* % error = 100 (antilog $s_M - 1$).

SUMMARY

The *in vitro* bioassay of corticotropin, based on the stimulation of the production of corticoids by isolated rat adrenals, has been modified to allow the use of the 4 point assay design and the factorial analysis of Bliss and Marks for the determination of the relative potency, and its error, of an unknown preparation. The method is rapid and convenient; an assay can be completed within a working day. The average index of precision, λ, of 24 assays was 0.15 ± 0.007. Of several pituitary preparations only the corticotropic preparations are significantly active in this test, indicating that the method is specific for corticotropic activity. The assays of corticotropin by the *in vitro* method are of the same order as those by the adrenal ascorbic acid depletion method of Sayers, Sayers and Woodbury.

Addendum

McKerns and Nordstrand (Can. J. Biochem. Physiol. **33**: 209. 1955.) have recently published an *in vitro* assay of corticotropin that differs in detail of design but yields similar results.

Acknowledgments

This investigation was supported by a Federal-Provincial Mental Health Grant, No. 604-5-12, to Dr. R. A. Cleghorn and by a grant from Canada Packers Limited.

The pituitary preparations were kindly supplied by Dr. Bruno Benfey and by the Armour Company, Nordic Biochemicals Limited, Frank W. Horner Limited, Connaught Medical Research Laboratories, and the United States Pharmacopoeial Convention, Inc.

Valuable criticism and statistical advice were furnished by Dr. J. W. Fisher, of the Mental Health Division, and by Dr. L. I. Pugsley and his colleagues, at the Food and Drug Division, of the Department of Health and Welfare, Ottawa.

We are indebted to Dr. R. A. Cleghorn for his encouragement during the course of this work.

REFERENCES

BIRMINGHAM, M. K., F. H. ELLIOTT AND P. H. L. VALERE: *Endocrinology* **53:** 687. 1953.

BLISS, C. I.: *Vitamin Methods.* Volume II, Academic Press, New York, 1951.

BLISS, C. I. AND H. P. MARKS: *Quart. J. Pharm. and Pharmacol.* **12:** 182. 1939.

ELLIOTT, F. H., M. K. BIRMINGHAM, A. V. SCHALLY AND E. SCHÖNBAUM: *Endocrinology* **55:** 721. 1954.

ELLIOTT, F. H. AND A. V. SCHALLY: *Can. J. Biochem. Physiol.* **33:** 174. 1955.

HOFFMANN, F. G. AND C. DAVISON: *Endocrinology* **54:** 580. 1954.

PUGSLEY, L. I.: *Endocrinology* **39:** 161. 1946.

SAFFRAN, M. and M. J. BAYLISS: *Endocrinology* **52:** 140. 1953.

SAFFRAN, M., B. GRAD AND M. J. BAYLISS: *Endocrinology* **50:** 639. 1952.

SAYERS, M. A., G. SAYERS AND L. A. WOODBURY: *Endocrinology* **42:** 379. 1948.

UMBREIT, W. W., R. H. BURRIS AND J. F. STAUFFER: *Manometric techniques and Tissue Metabolism.* 2nd Edition, Burgess, Minneapolis, 1949.

STEROID DIABETES IN GUINEA PIGS:

EFFECTS OF HYDROCORTISONE ADMINISTRATION ON BLOOD AND URINARY GLUCOSE, NITROGEN EXCRETION, FAT DEPOSITION, AND THE ISLETS OF LANGERHANS[1]

FRANZ X. HAUSBERGER AND ANDREW J. RAMSAY

Daniel Baugh Institute of Anatomy of the Jefferson Medical College of Philadelphia, Philadelphia, Pennsylvania

CORTISONE administration to guinea pigs with free access to food produces effects resembling in many respects Cushing's syndrome in man (Hausberger and Ramsay, 1953). The purpose of this study was to investigate the influence of hydrocortisone administration in guinea pigs. This steroid induces similar metabolic changes but the effects on body weight and nitrogen excretion are more marked than those produced by cortisone.

METHODS

Male guinea pigs weighing about 600 gm. were used. Body weight was recorded for several weeks prior to the experiment. The animals received greens daily in addition to Purina Rabbit checkers and water *ad lib.* After being placed in individual metabolic cages they were fed 50 gm. of lettuce daily, equivalent in protein content to 3 gm. checkers, and *ad lib.* weighed amounts of rabbit checkers, the amount of food consumed being determined by subtraction. Since most of the guinea pigs lost weight during the first days in the metabolic cage, analyses were started after they had regained their previous weight. Hydrocortisone acetate (Merck) was injected subcutaneously during the morning feeding but when given in equally divided dosages a second injection was administered in the late afternoon. Body weight, food intake, urinary glucose and non-protein nitrogen excretion were recorded every 24 or 48 hours. Urine was preserved by addition of citric acid and thymol and deproteinized before nitrogen determination with trichloroacetic acid. The amount of nitrogen excreted was divided by the amount of food consumed, the resultant being called in this study "the nitrogen food ratio." An increase of this ratio indicates an excess of nitrogen excretion, as outlined elsewhere (Hausberger and Ramsay, 1953).

Blood was obtained by puncturing the ear vessels. Blood glucose was determined according to Durham *et al.* (1950), urinary glucose according to Benedict (1911), and urinary non-protein nitrogen by the standard micro-Kjeldahl procedure. For analysis of the composition of adipose tissue a previously described method was used (Hausberger and Ramsay, 1953). For the histological evaluation of the islets, the pancreas was stained according to Gomori (1941).

Received for publication October 30, 1954.

[1] This investigation was supported by a research grant (A-345(C)) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service.

EXPERIMENTAL AND RESULTS

Eleven guinea pigs were injected at different dosage levels for 12 to 150 days. Five to 40 mg. of hydrocortisone per day were administered. The results shown in Figures 1–5 represent typical observations.

Body weight: All animals gained weight prior to the steroid administration. Six out of ten guinea pigs showed a retarded weight gain several days after starting the injections (Figs. 1, 2, 5). Four animals gained weight in a normal or almost normal rate during the first two to three weeks of the experiment proper (Figs. 3, 4) but thereafter the weight gain was also retarded. All animals treated for 4 weeks showed a considerable suppression of weight gain (Fig. 1) but an actual loss of body weight was observed in only one animal, the latter receiving 2×20 mg. of hydrocortisone per day (Fig. 5).

Although syringes and needles were sterilized daily, 5 out of 7 animals treated longer than 4 weeks developed infections at the site of the injections. The abscesses were opened and drained but during the time of the infection some temporary weight loss was observed. The appearance of infections was apparently not inhibited when 10 mg. of aureomycin were added twice weekly to the food. Three animals succumbed to deeper seated or general infections after a period of rapid weight loss.

The hydrocortisone administration was discontinued or interrupted in three guinea pigs after 78, 56, and 14 days, respectively. All animals showed temporary loss of weight and in the one which had received the steroid only for 14 days, the weight loss was for the shortest time but was

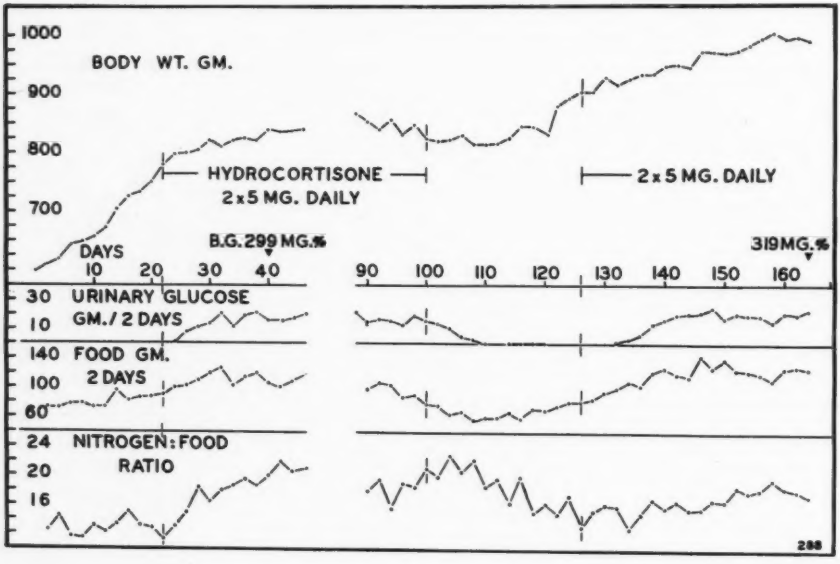
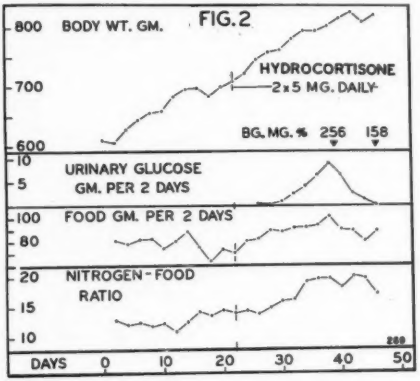
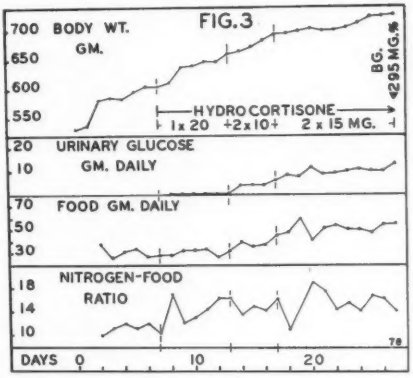
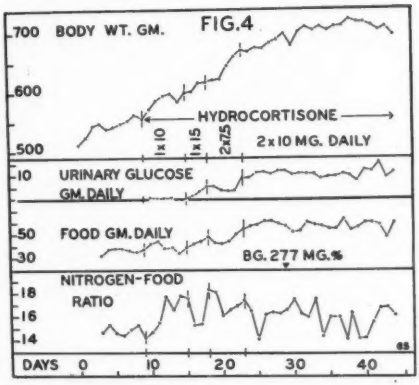
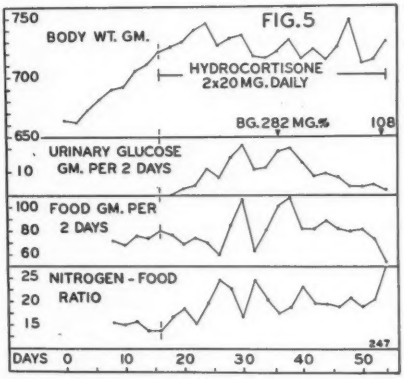


FIG. 1









most pronounced. After the period of loss, an increased weight gain could be observed throughout. When the injections were resumed in two of the guinea pigs, the weight curve behaved similar to that of the first period of the hydrocortisone administration (Fig. 1).

There was no evidence of increased water retention except in one animal (Fig. 5). All guinea pigs treated for at least four weeks and sacrificed in a generally healthy condition showed very large fat stores, the abdomen being filled with large masses of adipose tissue and the kidneys completely embedded in perirenal fat, whereas in untreated controls of similar weight about 1/3 to 1/2 of the anterior surface of these organs are free of fat. The weight of the two paired testicular fat bodies of fourteen controls was between 0.4 and 0.9% of the body weight while in the experimental animals the weight ranged from 0.9 to 1.33%. Furthermore, the testicular, renal, and subcutaneous (ex., inguinal) fat tissue of these guinea pigs showed a normal percentage of ether-acetone extractable lipids (90 to 95%). The animal described in Figure 5 had large subcutaneous edemas and the fat stores were rather small.

Food consumption: The increased or decreased food intake during the glucosuric period closely paralleled the loss of urinary glucose. The loss of calories due to the glucosuria was always fully compensated by the increased food intake. Only one animal (Fig. 5) retained and utilized less calories during the experimental period than during the pre-experimental one.

Glucosuria and blood glucose levels: The first traces of urinary sugar (0.1 to 0.4%) appeared usually on the fourth day. The blood sugar levels determined in two animals at this time were 122 and 158 mg. %. A significant elevation of the glucosuria and the blood glucose could only be achieved by injecting hydrocortisone twice daily, the most efficient dosage level being 5 mg. twice a day. Higher dosages did not accelerate the diabetic condition. On the contrary, when hydrocortisone was given in two 10 mg. dosages, or more, the animals often lost their appetites and the glucosuria and the blood glucose levels decreased. The highest glucose excretion was 18 gm. per day, determined from an animal which received 2×5 mg. of the steroid per day. At the peak of the glucosuria, blood glucose levels of 300 mg. % were observed. One animal, receiving 2×7.5 mg. of hydrocortisone, did not develop diabetes, and only traces of sugar (0.2 to 0.4%) were excreted occasionally during the time of the experiment while in another animal (Fig. 2), the glucosuria was only temporary.

A tendency of the diabetes to decrease considerably was seen in all animals that became ill and lost appetite. In such animals the blood glucose level dropped below 150 mg. %, while the urinary glucose concentration was still up to 2%. The excretion of urinary sugar at the time of normal blood glucose levels was especially pronounced in animals treated for several weeks, indicating that the effect on the kidney threshold for glucose seems to increase with the duration of the steroid administration. In two guinea pigs in which the hydrocortisone injections were resumed after 56 and 30 days of interruption, respectively, the urinary sugar excretion rose slower than during the first experimental period (Fig. 1). Three guinea pigs maintained diabetic for 3 to 5 months lost their glucosuria within 8 to 10 days after the treatment was discontinued.

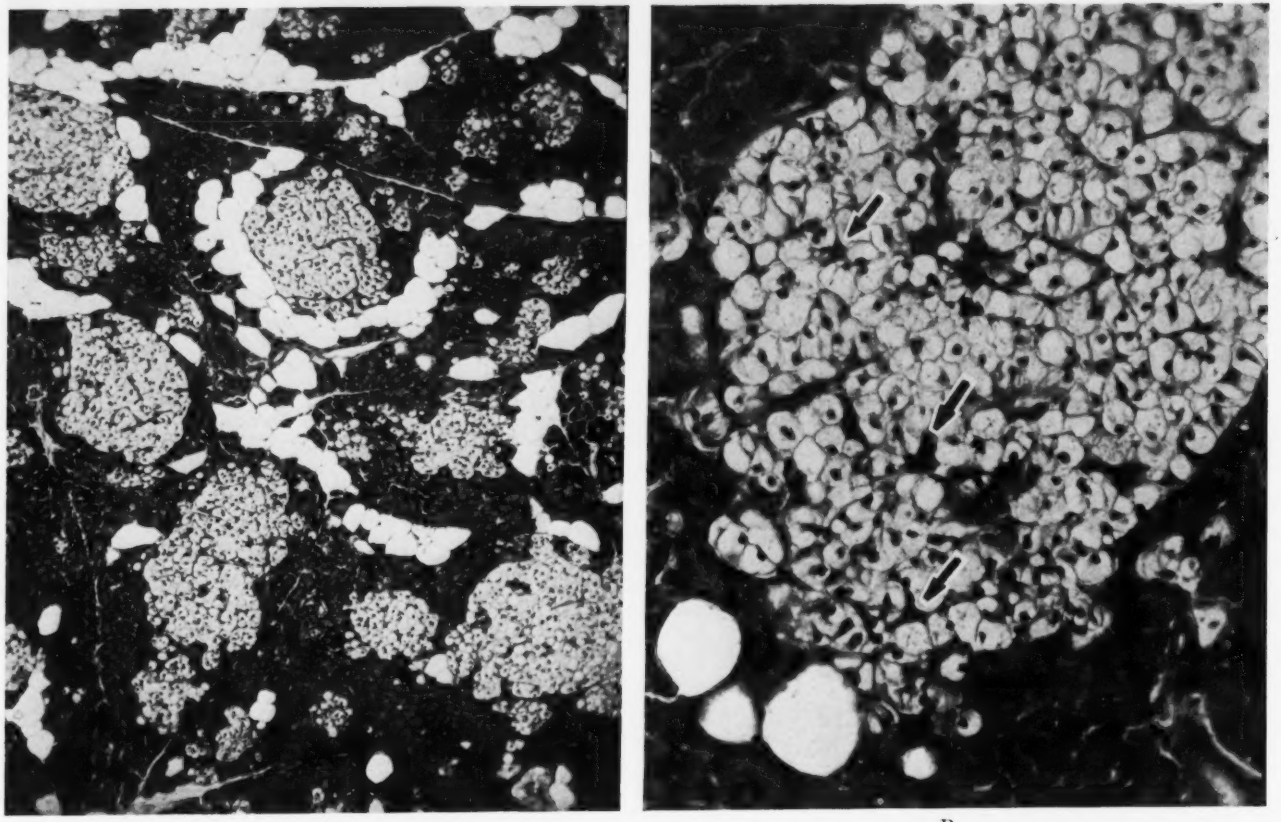
Urinary non-protein nitrogen: All hydrocortisone treated guinea pigs showed an excess loss of nitrogen, that is, the nitrogen-food ratio increased. The nitrogen loss was not related to the onset of the diabetes and there was little, if any, correlation with the weight gain or with the degree of the glucosuria. After the steroid administration was discontinued, the nitrogen excretion returned slowly to the norm (Fig. 1). The two animals, in which the steroid administration was resumed after a period of interruption, showed a slower increase of the nitrogen-food ratio than during the first experimental period (Fig. 1).

Adrenals, lymph nodes and thymus: The weight of the adrenals of 15 controls was between 0.050 and 0.081% of the body weight. The adrenals

of all guinea pigs treated with hydrocortisone showed atrophy of the cortex and weights ranged from 0.039 to 0.052% of the body weight. A definite dosage-time relationship could not be established, however, since the adrenals of the animal shown in Figure 1, for instance, weighed 0.052% of the body weight while other animals treated similarly, for shorter or longer times, showed more pronounced adrenal atrophy. The lymph nodes showed a considerable atrophy (to about 1/4 of the normal size). The cervical thymus, too, became rapidly atrophic but its weight was only moderately reduced in all those animals which received the corticosteroid for less than three weeks. Thereafter, however, the atrophic tissue was replaced by increased amounts of adipose tissue, so that the total weight of the cervical thymic lobes in most guinea pigs was found to be above the average of that of the controls.

Pancreas: The islets of the pancreas of all treated animals showed a marked hypertrophy. The size and number of the beta cells were considerably increased, and marked new formation of beta cells could be observed from centroacinar cells, ducts, and (perhaps) from acinar cells. The number of alpha cells was apparently not increased and they seemed normal in appearance, being present mainly in the larger and older islets. The newly formed smaller ones consisted almost entirely of beta cells. The beta cells of diabetic guinea pigs which were sacrificed in a healthy condition were considerably enlarged and completely or almost completely degranulated. The most marked change, severe hydropic degeneration, was observed in the islets of animals 288 and 326. The latter animal was maintained diabetic for $4\frac{1}{2}$ months, receiving 5 mg. of hydrocortisone twice daily, and excreting about 10 gm. of glucose daily, with a blood glucose level of around 300 mg.%. Nitrogen excretion and food intake was not followed in this animal. During the period of injections the weight increased slowly from 620 gm. to 960 gm., at which time the injections were discontinued, and the diabetes disappeared within 10 days. During the next two weeks a weight loss was observed which was followed by a rapid weight gain. The animal was sacrificed 33 days after discontinuing the steroid administration. The pancreas (Figs. 6, A and B) showed a remarkable amount of islet tissue. The hydropic beta cells were still not recovered, they were considerably enlarged with small and irregular nuclei. Only few apparently intact beta cells could be observed within the islets and between the acini. Unfortunately, no sugar tolerance tests were performed at this state.

Degranulation, enlargement, and new formation of beta cells as well as hypertrophy of the islets were much less marked in guinea pigs which fell ill during the time of hydrocortisone administration and which subsequently lost weight, appetite, and diabetes. An obviously increased storage of beta granulation could be seen in animals sacrificed after discontinuing the treatment and disappearance of the diabetes.



A B

FIG. 6A. Pancreas of a guinea pig diabetic for 135 days, shown 33 days after cessation of treatment. Note the great increase in size and number of islands. Much fat tissue has appeared between and within lobules. Gomori stain. ×70.

FIG. 6B. Island from the pancreas shown in Figure 6A. Note "hydropic" beta cells with shrunken and irregular nuclei, and clear cytoplasm bridged by reticulated condensations of cytoplasmic components. A few apparently normal alpha cells are seen. Arrows indicate small, compressed, beta cells with good granulation. Acinar tissue seems normal. Gomori stain. ×300.

DISCUSSION

Hydorcortisone administration in suitable dosage produces in guinea pigs, with free access to food, a steroid diabetes similar to that induced by cortisone. There are, however, qualitative differences. Most of the animals injected with hydrocortisone showed an early and often rather pronounced suppression of body weight gain. (Only one animal receiving 20 mg. of hydrocortisone twice daily developed edema.) The obesity seen in diabetic guinea pigs receiving hydrocortisone for a prolonged period of time was possibly less than in cortisone diabetic animals. The degree of the glucosuria was of similar magnitude in both groups but the blood glucose levels were lower in the hydrocortisone group, the values in this group being around 300 mg. % whereas in the cortisone diabetic animals values around 400 mg. %, were frequently observed. This difference may be due to the more pronounced effect of hydrocortisone on the kidney threshold of glucose. The most effective dosage for inducing diabetes seems to be 5 mg. of hydrocortisone twice daily. Only one animal in five receiving this dosage lost its diabetes while in the other four the diabetic condition could be maintained until the end of the experiment (in 3 animals up to four months). Guinea pigs injected with the same amount of cortisone always showed only a temporary diabetes. Higher dosages than twice 5 mg. of hydrocortisone did not produce a more severe diabetic condition. On the contrary such animals frequently lost their appetites and the diabetes decreased. Cortisone injections produced only occasionally a temporary and small loss of body nitrogen while hydrocortisone administration always caused a marked loss of excess nitrogen. Our experiments thus confirm the higher biologic activity of hydrocortisone as compared with that of cortisone (Ingle and Meeks, 1952).

The pronounced hypertrophy of the islet tissue suggests, as in cortisone treated animals, an increased insulin secretion which may be responsible for the increased appetite and the increased fat deposition. It is remarkable to note that high diabetic glucose levels maintained for several months, that is, about $\frac{1}{5}$ or $\frac{1}{6}$ of the life time of the guinea pig, do not produce a complete exhaustion of the beta cells. According to the concept advanced by Lukens (1941), 1944) and Dohan and Lukens (1948) one would expect that such prolonged hyperglycemia always induced a permanent diabetes. The pancreas of certain species, like that of guinea pigs, apparently has an almost inexhaustible regenerative power. The older beta cells degenerate but new ones are formed constantly in an amount sufficient to produce a fast disappearance of the diabetes after discontinuation of the steroid administration. The marked regenerative power of the pancreas of the guinea pig is also shown by the observation of Johnson (1949) that alloxan administration destroys practically all beta cells; still, a complete recovery of the islets takes place within a few days.

Experiments in progress will demonstrate whether still more prolonged steroid administration may cause a final exhaustion of the islets through destruction of the beta cells and the breakdown of their replacement mechanism.

SUMMARY

Male guinea pigs with free access to food, receiving hydrocortisone acetate twice daily by subcutaneous injection, develop a steroid diabetes which is characterized by pronounced hyperglycemia and glucosuria, reduced gain of body weight, greatly increased food intake, moderate obesity, and excess loss of nitrogen. The islets of the pancreas show marked hypertrophy, new beta cell formation and degranulation, followed, after prolonged administration of the steroid, by severe hydropic degeneration. The most effective dosage to induce a steroid diabetes was found to be 5 mg. of hydrocortisone given twice daily. Higher dosages produce frequently a loss of appetite and disappearance of the diabetes. A pronounced effect of hydrocortisone on the kidney threshold was observed. Although the diabetic condition was maintained up to more than four months, at which time the administration of the steroid was terminated, a permanent diabetes could not be induced.

REFERENCES

BENEDICT, S. R.: *J.A.M.A.* **57:** 1193. 1911.
DOHAN, F. C. AND F. D. W. LUKENS: *Endocrinology* **42:** 244. 1948.
DURHAM, W. F., W. L. BLOOM, G. T. LEWIS AND E. E. MANDEL: *Public Health Report* **65:** 670. 1950.
GOMORI, G.: *Am. J. Path.* **17:** 393. 1941.
HAUSBERGER, F. X. AND A. J. RAMSAY: *Endocrinology* **53:** 423. 1953.
INGLE, D. J. AND R. C. MEEKS: *Am. J. Physiol.* **170:** 77. 1952.
JOHNSON, D. D.: *Endocrinology*, **46:** 135. 1950.
LUKENS, F. D. W.: *Yale J. Biol. and Med.* **16:** 301. 1944.
LUKENS, F. D. W. AND F. C. DOHAN: *Endocrinology* **30:** 175. 1942.

EFFECTS OF RENIN IN RATS TREATED WITH METHYLANDROSTENEDIOL

G. M. C. MASSON, EDWIN R. FISHER, A. C. CORCORAN AND IRVINE H. PAGE

Research Division of the Cleveland Clinic Foundation and The Frank E. Bunts Educational Institute, Cleveland, Ohio

SKELTON (1953) reported that methylandrostenediol (MAD) caused mild hypertension and vascular lesions in uninephrectomized rats given 1% NaCl as drinking fluid. In comparison with DCA, MAD caused more renal hypertrophy and greater degenerative changes in the adrenal cortex. The purpose of this study was to examine further the hypertensive effect of MAD and to define the nature of the adrenal lesions. Previous studied have demonstrated that other steroids which elicit hypertension, namely DCA, cortisone and hydrocortisone, sensitize rats to the pressor and vasculotoxic effects of renin (Masson, Corcoran and Page, 1951, 1952; Masson, del Greco, Corcoran and Page, 1953). Similar studies were made in animals pretreated with MAD.

METHODS

Female albino rats were uninephrectomized and divided in 3 groups of 10 animals each; group averages of body weights were respectively 196, 202, and 202 grams. Groups I and II were given MAD over 90 days, during which group III served as untreated controls. The animals were maintained on Purina Fox Chow with 1% NaCl as drinking fluid. At the end of this time, each group was placed in metabolism cages and animals of Groups II and III were given renin subcutaneously over a course of 3 days. Blood was then withdrawn for determinations of urea nitrogen and serum proteins and the animals sacrificed. The tissues were examined by methods described below.

MAD (17α-methyl-Δ^5-androstene-3β, 17β-diol)[1] was given in a dose of 12.5 mg. of saline suspension, twice weekly for 1 month and 3 times weekly thereafter. Blood pressure was measured twice weekly (del Greco, Olmsted, Masson and Corcoran, 1953).

The renin preparation had an activity of 28 Goldblatt dog units per cc.; the total daily dose of 42 units was given subcutaneously in 3 divided doses. Fluid intake and urine flow were measured 1 day before and 2 days during treatment with renin.

RESULTS (TABLE I)

1. *MAD.* The average arterial pressure of the controls (Group III) ranged from 122 to 133 mm. of Hg during the 3 months of observation; the average

Received October 30, 1954.

This study was supported in part by a grant from the National Heart Institute, National Institutes of Health, Public Health Service.

[1] The authors are indebted to Dr. K. W. Thompson, Organon Inc., Orange, N. J., for a generous supply of Stenediol (Methylandrostenediol).

TABLE 1. PHYSIOLOGIC AND MORPHOLOGIC EFFECTS OF METHYLANDROSTENEDIOL ALONE OR IN COMBINATION WITH RENIN

		Group I	Group II	Group III
Treatment		MAD	MAD & Renin	Renin
Blood Pressure mm. Hg				
on day	0	131 (108–148)	123 (92–145)	122 (110–132)
	30	136 (120–150)	128 (100–147)	133 (123–152)
	60	153 (130–210)	134 (120–150)	131 (120–150)
	90	168 (150–200)	152 (120–210)*	128 (120–130)
Urine Flow cc./day				
on day	90	34 (18–60)	31 (13–66)	27 (15–55)
	94	30 (16–57)	86 (15–100)	64 (31–98)
Body Weight in gm.				
on day	90	259 (228–312)	269 (247–284)	232 (223–251)
	94	253 (224–320)	246 (214–289)	206–199–210)
BUN, mg. %		31 (26–34)	18.7 (10–23)	22.7 (20–23)
Serum Proteins, mg. %		4.35 (4.04–4.69)	3.44 (2.57–3.74)	3.68 (3.1–4.00)
Heart Weight, mg.		758 (580–952)	780 (625–920)	672 (530–725)
Kidney Weight, mg.		2172 (1972–2580)	1890 (1770–2390)	1410 (1220–1590)
Adrenals Weight, mg.		50.9 (40–57)	66.5 (49.6–69.6)	68.9 (64–78)

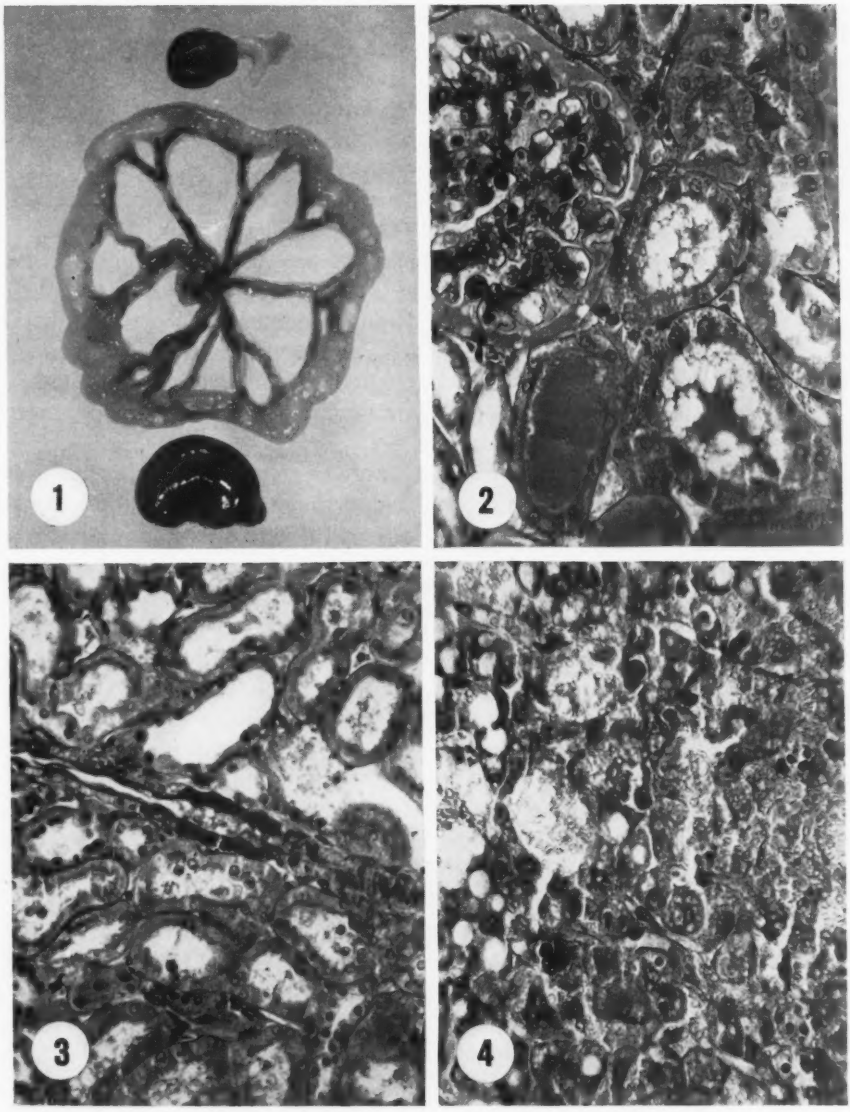
arterial pressures of the MAD-treated groups increased from 131 to 168 mm. Hg (Group I) and from 123 to 152 mm. Hg (Group II). Definite hypertension did not develop in all the MAD-treated animals; 3 of these showed pressures greater than 200 mm. Hg; pressures of from 170 to 200 mg. Hg were found in 4 at the end of the study.

Increased diuresis did not appear in the animals given MAD. One of the severely hypertensive animals showed hematuria.

2. *MAD and Renin.* Injection of renin in Groups II and III increased urine output and caused some decrease in body weight. No differences were observed between controls (Group III) and the animals pre-treated with MAD (Group II).

The average serum proteins and blood urea concentrations in both control and MAD-treated animals given renin, were lower than in the MAD-treated animals not given this agent.

3. *Structural Changes.* The adrenals of rats given renin were larger than those of the MAD-treated controls (Group I). Otherwise, the structural changes in Groups I and II were so similar that they are considered together as representing the effects of MAD. Thus, both Groups I and II showed definite increases in kidney weight and slight increases in heart weight. There was no gross edema in any of the rats; slight peritoneal effusions were found only in 2 animals of Group I and in 1 animal of Group II and were associated with thickening of mesenteric and pancreatic arteries (Fig. 1). These 3 animals with serous effusions showed also the largest kidneys and had the highest blood pressure (over 200 mm. Hg). The kidneys of these, and to a lesser degree, those of other animals given MAD, were pale



FIGS. 1–4. MORPHOLOGIC CHANGES IN METHYLANDROSTENEDIOL-TREATED RATS.

FIG. 1. Mesenteric arteritis and enlargement of kidney and heart.

FIG. 2. Kidney showing hypertrophy of cells and focal deposits of hyalin substance in glomerulus, and, retention of glomerular filtrate; tubular hyalin casts nnd ath-locytosis of hyalin droplets.

FIG. 3. Focal arteriolar hyalinization in kidney.

FIG. 4. Cellular colloid degeneration and vacuolization in adrenals.

yellow and the surfaces were irregularly marked with white or red points. The ovaries of animals given MAD were uniformly atrophic and uterine size increased.

Lesions attributable to MAD hypertension are described as they were seen in animals most severely affected. In these, the kidneys showed glomerular and tubular changes. The glomeruli were enlarged, the capillary basement membrane thickened and fragmented, with swollen pericytes, some of which contained PAS-positive droplets; the capsular space was enlarged and contained a substance which, like plasma, stained lightly with PAS. The proximal convoluted tubules showed focal dilatation and their epithelial cells, hyaline droplet formation; hyaline casts occurred in distal and collecting tubules (Fig. 2). A slight lymphocytic infiltrate surrounded some of the tubules involved in these changes. The distribution of alkaline phosphatase was not abnormal. The tubular changes were doubtfully more severe in the animals given renin.

The pancreatic and mesenteric vessels of the severely hypertensive animals showed the arteritis (necrosis, hemorrhage, edema, inflammatory exudate) which characterizes hypertensive disease in the rat. Comparable lesions were found in the heart and kidney (Fig. 3).

The adrenal effect of MAD (Fig. 4) was manifested in generalized cortical lipid depletion and focal cellular fatty metamorphosis. Cells of the zona fasciculata contained homogenous, spherical inclusions surrounded by a clear halo and which ranged up to 10 microns in diameter. Occasionally, several such bodies were observed within a single cell. The various histochemical tests indicate that these inclusions are composed of glycoprotein or mucoprotein. This characterization is based on the following histochemical observations: periodic acid-Schiff (PAS) intensively positive, unaffected by diastase digestion; Rinehart-Abul-Haj, positive, not affected by hyaluronidase nor metachromatic to thiazine dye; Millon, diazonium-coupling, ninhydrin-Schiff positive; ferric ferricyanide positive, unaffected by saturated mercuric chloride (which would eliminate sulfhydryl as the reacting group); marked affinity for acid dyes; reactions to oil red 0 and other sudanophilic dyes, Baker acid hematin, Fischler's method for fatty acid, peracetic acid-Schiff and plasmal reaction were negative. Loss of lipid from the cells and absence thereof from the intracellular inclusions was indicated by the latter series of lipid stains, and also by loss of birefringence from the cortical sections.

DISCUSSION

The data confirm the fact that MAD can evoke hypertension and hypertensive vascular lesions in rats. They also add weight to Skelton's suggestions (1953) that this hypertensive process may have more in common with hypertension elicited by lyophilized anterior pituitary (LAP) than with DCA hypertension; this is indicated by the wide scatter of the blood pres-

sures of animals treated with MAD, as well as LAP (Masson, Corcoran and Page, 1949), the absence of the polyuria associated with DCA (Masson, Corcoran and Page, 1949) and the similarities of responses of kidney, heart and adrenal tissues to MAD and LAP (Selye and Stone, 1950). A genetic difference between MAD and DCA hypertension is also indicated by the failure of renin to elicit, in MAD-treated animals, the vasculotoxic state which it provokes in rats pre-treated with DCA, cortisone and hydrocortisone (Masson, Corcoran and Page, 1951, 1952; Masson, del Greco, Corcoran and Page, 1953). Somatotrophin is presumably the major active component in LAP; like MAD, somatotrophin does not sensitize to renin (Masson, del Greco, Corcoran and Page, 1953). Lastly, MAD is known to have no adrenal cortical replacement activity; rather, the adrenal cortical changes it elicits indicate depletion and degeneration; the histochemistry of the cellular inclusions suggests that these would not have cortical hormonal activity.

The three agents, other than steroids with adrenal cortical activity, which elicit hypertension in the rat (LAP, somatotrophin and MAD) (Masson, Corcoran and Page, 1949; Selye, 1951) have in common the property of causing kidney growth. These experimental hypertensions therefore bear directly on Braun-Menendez's hypothesis (1952) that the ultimate stimulus to renal hypertension is a "renotrophin," evoked by some situation which provokes a stimulus to kidney growth and which becomes pressor if the kidney is prevented from responding fully to the stimulus. Renin has been found to inhibit compensatory renal hypertrophy (Schaffenburg, Masson and Corcoran, 1954). This suggests the possibility that an untoward renotrophic substance, such as MAD, LAP or somatotrophin, may provoke a homeostatic release of renin which would act as an anti-renotrophin. The renin thus released could then cause renal hypertension. The indirect nature of this pressor action of renotrophic agents, may account for the variability with which these elicit experimental hypertension.

CONCLUSIONS

Methylandrostenediol is shown to elicit hypertension and hypertensive vascular lesions in uninephrectomized, salt-fed rats. This hypertensive state resembles the type of hypertension produced by injections of anterior pituitary extracts; both are associated with degenerative changes in the adrenals. The droplets which form in the adrenal cortex react histochemically as muco- or glyco-proteins.

Unlike desoxycorticosterone or the adrenal steroids, cortisone and hydrocortisone, MAD does not sensitize to the acute vasculotoxic effects of subcutaneously injected renin.

The mechanism of MAD hypertension is distinct from that of DCA hypertension. It may represent an indirect response, possibly mediated by renin, to the stimulus of excessive renal growth.

REFERENCES

BRAUN-MENENDEZ, E.: *Am. physiol. latino Am.* **2:** 2. 1952.

GRECO, DEL, F., F. OLMSTED, G. M. C. MASSON AND A. C. CORCORAN: *J. Lab. and Clin. Med.* **41:** 729, 1953.

MASSON, G. M. C., A. C. CORCORAN AND I. H. PAGE: *J. Lab. and Clin. Med.* **34:** 1416. 1949.

MASSON, G. M. C., A. C. CORCORAN AND I. H. PAGE: *J. Lab. and Clin. Med.* **38:** 213. 1951.

MASSON, G. M. C., A. C. CORCORAN AND I. H. PAGE: *A.M.A. Arch. Path.* **53:** 217. 1952.

MASSON, G. M. C., F. DEL GRECO, A. C. CORCORAN AND I. H. PAGE: *A.M.A. Arch. Path.* **56:** 23. 1953.

MASSON, G. M. C., F. DEL GRECO, A. C. CORCORAN AND I. H. PAGE: *Am. J. Med. Sc.* **226:** 296. 1953.

SCHAFFENBURG, C., G. M. C. MASSON AND A. C. CORCORAN: *Proc. Soc. Exptl. Biol. and Med.* **87:** 469. 1954.

SELYE, H. AND H. STONE: On the morphology of the adrenal cortex. American Lecture Series, number 74, 1950, Charles C Thomas, Springfield.

SELYE, H.: *Brit. Med. J. i:* 263. 1951.

SKELTON, F. R.: *Endocrinology* **53:** 492. 1953.

THE ROLE OF THE PITUITARY AND THYROID GLANDS IN THE PHOSPHORUS METABOLISM OF THE ADRENAL GLAND DURING COLD STRESS[1]

DORIS NICHOLLS AND R. J. ROSSITER

Department of Biochemistry, University of Western Ontario, London, Canada

REISS and Halkerston (1950) showed that if rats were exposed to a cold environment for a short period of time there was an increase in the incorporation of inorganic phosphate labeled with radioactive phosphorus (P^{32}) into the total acid-soluble fraction of the adrenal gland. This finding was confirmed by Nicholls and Rossiter (1955), who also showed that their was an increase in the incorporation of P^{32} into both the inorganic phosphate and the easily-hydrolyzable acid-soluble phosphorus of the adrenal gland. If rats were exposed to the cold for longer periods of time, the increase in the P^{32} incorporation was biphasic. The first response was greater in magnitude and was maximal after 2 or 3 hours in the cold, followed by a return almost to normal by 24 hours. The second response did not occur until the animals had been maintained in the cold for several days.

Since both the injection of ACTH (Gemzell and Samuels, 1950; Riedel, Logan, DeLuca and Rossiter, 1954) and exposure of the animals to the cold for short periods of time (Reiss and Halkerston, 1950; Nicholls and Rossiter, 1955) caused an increase in the incorporation of P^{32} into the acid-soluble phosphorus of the adrenal, it was suggested that the early phase might indicate an increase in the metabolism of the adrenal brought about by release of endogenous ACTH from the pituitary gland (Nicholls and Rossiter, 1955). The experiments reported below support this view, since the increased P^{32} incorporation following a short exposure to the cold was eliminated by removal of the pituitary gland.

The possibility was also considered that the increase in the incorporation of P^{32} into the acid-soluble phosphorus fractions of the adrenal gland might be due to an increased activity of the thyroid, since the changes in the adrenal were in the same direction as those reported for muscle (Greenberg, Fraenkel-Conrat and Glendening, 1947) and liver (Venkataraman, Venkataraman, Schulman and Greenberg, 1950) during hyperthyroidism produced by the administration of dried thyroid. Such an explanation might well apply to the later phase of increased P^{32} incorporation, since there is good evidence that the thyroid gland is more active after an animal

Received for publication October 30, 1954.

[1] This work was supported by a grant from the Defence Research Board of Canada.

has been exposed to the cold for a period of days (Dempsey and Astwood, 1943; Leblond, Gross, Peacock and Evans, 1944; Sellers and You, 1950; Rand, Riggs and Talbot, 1952; Catz, Rawi and Geiger, 1953). The experiments reported below show that when thyroid function was depressed with thiouracil, the increase in the incorporation of P^{32} into the acid-soluble phosphorus fractions of the adrenal, brought about by short exposures (3 hours) to the cold, was unimpaired. The response after longer periods in the cold (8 days), although still present, was considerably decreased.

METHODS

Hypophysectomized Rats

Hypophysectomized male rats of the Sprague-Dawley strain were obtained from the Hormone Assay Laboratories, Chicago. Microscopic examination of the pituitary region of representatives of these animals revealed no evidence of remaining pituitary tissue. The adrenal glands showed the typical microscopic changes associated with hypophysectomy, including atrophy and an increase in cholesterol as determined by the Schultz method. Observations on the effect of 3 hours in the cold room ($3 \pm 1°$ C.) were made on these animals, either 2 or 14 days after removal of the pituitary, and on normal male rats of similar strain and age. In the cold-stressed animals the P^{32} uptake was measured for the 3 hours during which the animals were in the cold room. In the controls the P^{32} uptake was measured for 3 hours at room temperature ($22 \pm 1°$ C.). The animals were allowed free access to food (Master Fox Cubes, Toronto Elevators, Ltd.).

Thiouracil-treated Rats

The antithyroid drug, 2-thiouracil, was administered as a 0.1% solution in the drinking water, which was supplied in unlimited quantities, for two weeks to young male rats of the Sprague-Dawley strain. This assured that the animals received a daily dosage of thiouracil in excess of that required for a maximal histological effect on the thyroid gland (Astwood, 1943). The uptake of P^{32} was measured on these animals and on untreated rats of similar strain and age. The cold-stressed animals were placed in the cold room ($3 \pm 1°$ C.) for three hours, and the controls were studied at room temperature ($22 \pm 1°$ C.) as described for the hypophysectomized animals.

Rats treated with thiouracil (MacBeth and Noble, 1949; Sellers and You, 1950), like thyroidectomized rats (Ring, 1939; Leblond and Gross, 1943), do not survive extended exposure to the cold. To enable the thiouracil-treated animals to survive for longer periods of time, a daily dose of 2.5 μg. thyroxine in alkaline saline solution was administered subcutaneously, commencing at the time of the thiouracil treatment, i.e. six days before the animals were exposed to the cold. Thyroxine was similarly administered to a group of animals not receiving thiouracil. The effect of 8 days in the cold was studied in these two groups and also in a group of untreated animals.

The incorporation of P^{32} into the acid-soluble P fractions of the adrenal glands of the rats of both thiouracil experiments (3 hours or 8 days in cold) was measured for 2 hours during the time the animals were in the cold room for the cold-stressed rats and for 2 hours at room temperature in controls. This is in contrast to the hypophysectomy experiments, where the uptake time was 3 hours. All the animals were allowed free access to food.

A histological examination of the thyroid glands of a representative group of thiouracil and/or thyroxine treated animals showed that the treatment had been effective. The administration of thyroxine led to the accumulation of colloid in the gland and to

atrophic changes in the follicular lining. Thiouracil treatment produced thyroid hyperplasia, characterized by small follicles, almost empty of colloid, and lined by low columnar epithelium. The hyperplastic changes resulting from thiouracil treatment were partially prevented by thyroxine administration.

Specific Activity Measurements

Observations were made on the effects of cold stress on the concentration of P and the incorporation of P^{32} into the following P-containing fractions: the inorganic P of the plasma, the inorganic P, 20-minute hydrolyzable P (consisting mainly of the labile P of ATP), and total acid-soluble P of the adrenal gland. The specific activity (cts./min./μg. P) of each of the adrenal fractions was expressed as a percentage of that of the plasma (relative specific activity). The specific activity of the plasma inorganic P for each animal was adjusted to a standard injection of 200 μc. per 200 gm. body weight, based on a simulated P^{32} standard. Details of the experimental procedure have been described elsewhere (Nicholls and Rossiter, 1955).

RESULTS

Hypophysectomy

Table 1 shows the specific activity of the plasma inorganic P and the relative specific activities of the adrenal fractions of normal animals and hypophysectomized animals studied either 2 or 14 days after removal of the pituitary. In each instance figures are given for control animals kept at room temperature and for experimental animals exposed to the cold for 3 hours.

In the animals maintained at room temperature, the specific activity of the plasma inorganic P of the hypophysectomized animals studied 2 days after the operation was significantly ($P < 0.001$) increased and was still further increased 14 days after the operation. The relative specific activity of each of the adrenal fractions was significantly ($P < 0.05$–0.001) decreased following hypophysectomy. These findings confirm previous reports (Gemzell and Samuels, 1950; Geschwind, Li, and Evans, 1951; Riedel, *et al.*, 1954; Riedel and Rossiter, 1954). In the rats exposed to the cold for three hours the effect of hypophysectomy was similar to that observed for the animals maintained at room temperature. The mean specific activity of plasma inorganic P of the hypophysectomized group was significantly ($P < 0.001$) greater than that of the normal group and the mean relative specific activity of each of the adrenal fractions was significantly ($P < 0.001$) less.

In the normal animals the specific activity of the plasma inorganic P was not significantly changed by a 3-hour exposure to the cold, but there was a significant ($P < 0.01$) increase in the relative specific activity of each of the adrenal fractions, thus confirming work already reported (Nicholls and Rossiter, 1955).

Removal of the pituitary completely abolished the increase in the relative specific activity of the total acid-soluble P of the adrenal after an exposure to the cold of 3 hours. This was true for animals studied either 2 or

TABLE 1. EFFECT OF EXPOSURE TO THE COLD FOR 3 HOURS ON THE INCORPORATION OF P^{32} INTO THE ACID-SOLUBLE P FRACTIONS OF THE ADRENAL GLAND OF NORMAL AND HYPOPHYSECTOMIZED RATS

	Specific activity[1] (cts./min./μg. P)			Relative specific activity ×10²								
	Plasma inorg. P			Adrenal inorg. P			20-min. hydrolyzable P			Total acid-soluble P		
	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold
Normal	254±9* (7)†	>0.4	246±5 (6)	90.8±13.3 (7)	<0.01	148±6.4 (7)	50.1±7.5 (6)	<0.01	83.6±5.4 (7)	49.8±8.3 (7)	<0.01	79.9±3.1 (7)
P[1]	<0.001		<0.001	<0.001		<0.001	<0.05		<0.001	<0.01		<0.001
Hypophysectomized 2 days	384±12 (10)	>0.1	376±12 (11)	46.1± 1.2 (10)	<0.05	53.0±2.5 (11)	31.9±4.0 (10)	>0.2	27.4±1.2 (10)	24.8±1.1 (10)	>0.7	24.4±0.9 (11)
Hypophysectomized 14 days	499±27 (5)	<0.02	610±25 (5)	59.7±4.0 (5)	>0.1	49.2±4.0 (5)	23.9±1.0 (5)	<0.05	28.3±1.3 (5)	29.9±2.0 (5)	>0.1	25.9±1.4 (5)

* Mean ± standard error.
† Number of rats.
[1] P value for hypophysectomized rats (2 days after operation) compared with normals.
[2] P value for rats in the cold compared with controls at room temperature.
[i] Injected dose, 4.3×10^7 cts./min. for a 200 gm. rat.

TABLE 2. EFFECT OF HYPOPHYSECTOMY ON THE CONCENTRATION OF THE INORGANIC P OF THE PLASMA AND THE ACID-SOLUBLE P OF THE ADRENAL OF THE RAT

	P concentration			
	Mg./100 ml.	Mg./100 gm.		
	Plasma inorg. P	Adrenal inorg. P	20-min. hydrolyzable P	Total acid-soluble P
Normal	7.18±0.27* (14)†	19.0±0.80 (14)	23.5±0.95 (14)	94.2±1.01 (14)
P[1]	<0.001	>0.3	<0.01	<0.001
Hypophysectomized 2 days	5.57±0.17 (21)	17.7±1.08 (21)	19.1±1.08 (21)	77.6±1.21 (21)
P[2]	<0.001	>0.3	<0.001	<0.001
Hypophysectomized 14 days	4.32±0.47 (10)	18.0±0.71 (10)	16.5±0.93 (10)	69.3±0.61 (10)

* Mean ± standard error.
† Number of rats.
[1] P value for hypophysectomized rats 2 days after operation compared with normals.
[2] P value for hypophysectomized rats 14 days after operation compared with normals.

14 days after the operation. For the 2-day animals the relative specific activity of the 20-minute hydrolyzable P was not increased and that of the inorganic P showed an increase that was just significant statistically ($P < 0.05$). However, the increase (18%) was small compared with that (67%) for the normal animals. For the 14-day animals the relative specific activity of the inorganic P was not increased and that of the 20-minute hydrolyzable P was increased by only 15% ($P < 0.05$), compared with a 63% increase in the normal animals.

The 3-hour exposure to the cold caused no significant changes in the concentration of inorganic P in the plasma, or in the concentration of P in each of the three adrenal acid-soluble P fractions, thus confirming previous findings (Nicholls and Rossiter, 1955). However, the data, presented in Table 2, illustrate clearly the effect of hypophysectomy on the concentration of P in these fractions. Two days after the removal of the pituitary there was a significant ($P < 0.001$) fall in the concentration of inorganic P in the plasma, and a further fall by 14 days. This confirms previous findings by a number of workers (summarized by Riedel *et al.*, 1954). The decrease in the concentration of total acid-soluble P ($P < 0.001$) and the failure to observe a significant decrease in the concentration of adrenal inorganic P ($P > 0.3$) after hypophysectomy confirms the report of Riedel *et al.* (1954). Table 2 shows that the decrease in the concentration of total acid-soluble P is in part due to the significant ($P < 0.001$) decrease in the concentration of 20-minute hydrolyzable P previously reported by Riedel and Rossiter (1954).

Thiouracil-Treatment—3-Hour Exposure to Cold

Table 3 shows that treatment with thiouracil for 2 weeks caused an increase in the weight of the thyroid gland similar to that described by Astwood (1943), and a decrease in the weight of the adrenal gland. This decrease was still significant ($P < 0.001$) when the adrenal weight was calculated in terms of 100 gm. body weight. Decreases in adrenal weight follow-

TABLE 3. EFFECT OF THIOURACIL TREATMENT FOR 2 WEEKS ON THE WEIGHT OF THE THYROID AND ADRENAL GLANDS OF THE RAT

	Thyroid weight		Adrenal weight	
	Mg.	Mg./100 gm.	Mg.	Mg./100 gm.
Normal	11.8 ± 0.30* (20) †	6.79 ± 0.70 (20)	31.7 ± 0.82 (20)	18.2 ± 0.39 (20)
P[1]	<0.001	<0.001	<0.001	<0.001
Thiouracil	21.2 ± 0.95 (28)	14.6 ± 0.79 (28)	23.4 ± 0.37 (28)	15.9 ± 0.37 (28)

* Mean ± standard error.
† Number of rats.
[1] P value for thiouracil-treated rats compared with normals.

[2] P value for hypophysectomized rats 14 days after operation compared with normals.

ing either the administration of thiouracil or thyroidectomy have been described (Leblond and Gross, 1943; Leblond and Hoff, 1944; Baumann and Marine, 1945; Freedman and Gordon, 1950, and others), but there are some discrepancies in the reported findings (see reviews by Zarrow and Money, 1949; Perry, 1952; and Hess, 1953). Apparently, if older rats are used, or if the calorie intake of the controls is restricted so that they are maintained at a body weight similar to that of the hypothyroid animals, there is no significant fall in adrenal weight. In addition, it should be noted that thiouracil causes a hypertrophy of the medulla of the adrenal gland (Marine and Baumann, 1945).

Table 4 shows the effect on the P^{32} incorporation of exposing normal and thiouracil-treated animals to the cold for 3 hours. In control animals maintained at room temperature, thiouracil caused a significant ($P < 0.01$) increase in the specific activity of the plasma inorganic P. This confirms the findings of others (Greenberg *et al.*, 1947; Flock, Bollman, and Berkson, 1948; and Venkataraman *et al.*, 1950). There was a significant ($P < 0.001$) decrease in the relative specific activity of each of the adrenal fractions. This finding was not unexpected, for Boatman, Sunder, Russ and Moses (1951) reported that thyroidectomy decreased the incorporation of total P^{32} into the adrenal gland. In the animals exposed to the cold, thiouracil caused changes similar to those found for the animals maintained at room temperature, i.e. an increase in the specific activity of the plasma inorganic P and a decrease in the relative specific activity of each of the adrenal fractions.

In the normal animals a 3-hour exposure to the cold caused no significant change in the specific activity of the inorganic P of the plasma, but the relative specific activity of each of the adrenal fractions was significantly ($P < 0.001$) higher. The results are essentially the same as those of the experiment reported in Table 1, where the animals were killed 3 hours rather than 2 hours after the injection of the P^{32}.

In the thiouracil-treated animals the cold exposure caused an increase in the specific activity of the plasma inorganic P that was just significant statistically ($P < 0.05$). Such a tendency was noted previously in the 14-day hypophysectomized rats exposed to cold for 3 hours (Table 1). The relative specific activity of each of the adrenal fractions was significantly ($P < 0.001$) increased in the cold. In fact, the response in the adrenals of the thiouracil-treated rats to a 3-hour exposure to cold was as great as, or greater than, that observed in the normal animals.

Thiouracil-Treatment—8-Day Exposure to Cold

Table 5 shows the effects of the administration of thyroxine or thyroxine plus thiouracil on the weights of the thyroid and adrenal glands of rats maintained at room temperature for 2 weeks, or maintained at room temperature for 6 days and then exposed to the cold for 8 days. In control ani-

TABLE 4. EFFECT OF EXPOSURE TO THE COLD FOR 3 HOURS ON THE INCORPORATION OF P^{32} INTO THE ACID-SOLUBLE P FRACTIONS OF THE ADRENAL GLAND OF NORMAL AND THIOURACIL-TREATED RATS

	Specific activity[I] (cts./min./μg. P)			Relative specific activity								
	Plasma inorg. P			Adrenal inorg. P			20-min. hydrolyzable P			Total acid-soluble P		
	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold	Room temp.	P[2]	Cold
Normal	302±9* (25)†	>0.6	296±9 (27)	66.3±3.1 (28)	<0.001	100±3.5 (29)	31.1±0.4 (24)	<0.001	58.3±2.2 (26)	29.5±1.4 (27)	<0.001	51.9±2.6 (27)
P[1]	<0.01		<0.001	<0.001		<0.001	<0.001		<0.001	<0.001		<0.001
Thiouracil	358±14 (13)	<0.05	413±18 (14)	43.7±2.6 (14)	<0.001	78.6±4.5 (13)	23.3±1.3 (14)	<0.001	43.8±3.8 (12)	17.0±1.0 (14)	<0.001	35.2±2.8 (12)

* Mean ± standard error.
† Number of rats.
[1] P value for thiouracil-treated rats compared with normals.
[2] P value for rats in the cold compared with controls at room temperature.
[I] Injected dose, 4.3×10^7 cts./min. for a 200 gm. rat.

TABLE 5. EFFECT OF 8 DAYS IN THE COLD ON THE WEIGHT OF THE THYROID AND ADRENAL GLANDS OF NORMAL RATS AND BOTH NORMAL AND THIOURACIL-TREATED RATS RECEIVING THYROXINE

	Thyroid weight						Adrenal weight					
	Mg.			Mg./100 gm.			Mg.			Mg./100 gm.		
	Room temp	P[3]	Cold	Room temp	P[3]	Cold	Room temp	P[3]	Cold	Room temp	P[3]	Cold
Normal +Thyroxine	11.7±0.46* (16)†	<0.01	9.99±0.43 (16)	6.11±0.23 (16)	>0.6	5.99±0.20 (16)	31.9±0.84 (16)	<0.001	40.0±1.02 (16)	16.7±0.48 (16)	<0.001	24.3±0.90 (16)
P[1]	>0.4		>0.1	>0.05		>0.3	>0.7		>0.8	>0.1		>0.5
Normal	11.2±0.41 (17)	>0.4	10.8±0.37 (16)	5.67±0.11 (17)	<0.01	6.24±0.20 (16)	31.5±1.10 (17)	<0.001	40.4±1.24 (16)	15.9±0.39 (17)	<0.001	23.4±1.04 (16)
P[2]	<0.001		<0.001	<0.001		<0.001	<0.01		<0.001	>0.4		>0.1
Thiouracil +Thyroxine	17.0±0.82 (16)	>0.05	14.9±0.75 (17)	9.30±0.41 (16)	>0.8	9.14±0.59 (17)	27.9±0.80 (16)	<0.001	34.6±0.94 (17)	15.4±0.56 (16)	<0.001	21.1±1.03 (17)

* Mean ± standard error.
† Number of rats.
[1] P value for normal rats receiving thyroxine compared with normals.
[2] P value for thiouracil-treated rats receiving thyroxine compared with normals.
[3] P value for rats in the cold compared with controls at room temperature.

mals maintained at room temperature, treatment with thiouracil plus thyroxine caused a significant ($P < 0.001$) increase in thyroid weight, both on an absolute basis and calculated in terms of 100 gm. body weight, although the increase was not as great as previously reported for rats that did not receive thyroxine (cf. Table 3). This finding is in agreement with the observations of Dempsey and Astwood (1943), that thyroxine is able to counteract the thiouracil-induced increase in thyroid weight. The room-temperature animals receiving thyroxine alone showed no significant change in thyroid weight.

In the control animals maintained at room temperature treatment with thyroxine and thiouracil also caused a significant ($P < 0.01$) decrease in adrenal weight, although not to the same extent as previously found for rats that did not receive thyroxine (cf. Table 3). This observation is in agreement with the report of Freedman and Gordon (1950) that thyroxine is able to reverse the fall in adrenal weight induced by thiouracil. No significant changes were found in the adrenal weight relative to the body weight. In the animals treated with thyroxine alone there was no significant change in adrenal weight. In the rats exposed to the cold for 8 days treatment with thiouracil caused changes in the adrenal and thyroid weights that were in the same directions as those reported for the animals maintained at room temperature.

Exposure to the cold for 8 days did not cause a significant increase in the mean weight of the thyroid gland in any of the groups studied. However, on a body weight basis, the thyroid weight of the normal animals was increased significantly ($P < 0.01$), thus confirming the reports of Kenyon (1933) and Rand *et al.* (1952). Exposure to the cold for 8 days caused no significant increase in the thyroid weight relative to body weight of animals treated with thiouracil and thyroxine. Previously Dempsey and Astwood (1943) and Rand *et al.* (1952) showed that exposure to the cold caused an increase in the thyroid weight of rats receiving thiouracil and small daily doses of thyroxine or dried thyroid. The results reported in Table 5 are not comparable with these findings, however, for the experimental conditions differed in a number of important respects.

The 8-day exposure to the cold caused a significant ($P < 0.001$) increase in the adrenal weight of normal rats, as well as in that of rats receiving thiouracil and thyroxine, or thyroxine alone. The increase in adrenal weight of normal rats exposed to cold is well known (for references, see Nicholls and Rossiter, 1955). Leblond and Gross (1943) reported that thyroidectomy did not abolish this response.

Table 6 shows the incorporation of P^{32} into the plasma inorganic P and the adrenal acid-soluble P fractions. In control animals not exposed to the cold, there were no significant changes in the specific activity of the plasma inorganic P or in the relative specific activities of the adrenal fractions attributable to treatment with thiouracil plus thyroxine or thyroxine alone.

TABLE 6. EFFECT OF 8 DAYS IN THE COLD ON THE INCORPORATION OF P^{32} INTO THE ACID-SOLUBLE P FRACTIONS OF THE ADRENAL GLAND OF NORMAL RATS AND BOTH NORMAL AND THIOURACIL-TREATED RATS RECEIVING THYROXINE

	Specific activity[I] (cts /min /μg P)			Relative specific activity								
	Plasma inorg P			Adrenal inorg P			20-min hydrolyzable P			Total acid-soluble P		
	Room temp	P[3]	Cold	Room temp.	P[3]	Cold	Room temp.	P[3]	Cold	Room temp.	P[3]	Cold
Normal +Thyroxine	289±9* (13)†	>0.05	252±16 (14)	53.7±1.8 (14)	<0.001	73.4±4.6 (13)	28.2±1.1 (12)	<0.001	42.5±2.8 (11)	23.7±1.2 (14)	<0.01	32.3±2.5 (12)
P[1]	>0.3		>0.3	>0.3		>0.7	>0.1		>0.7	>0.2		>0.3
Normal	274±13 (16)	<0.05	233±14 (16)	58.3±4.4 (15)	<0.01	75.4±3.6 (15)	34.3±4.1 (13)	<0.05	46.3±3.8 (13)	27.4±2.9 (15)	<0.05	35.8±2.4 (15)
P[2]	>0.05		<0.001	>0.3		<0.01	>0.1		<0.05	>0.05		<0.001
Thiouracil +Thyroxine	308±13 (16)	>0.4	291±15 (13)	53.7±2.1 (16)	>0.05	60.1±2.4 (13)	27.7±1.1 (14)	<0.01	34.8±2.3 (11)	21.9±0.9 (16)	<0.01	25.8±2.8 (13)

* Mean ± standard error.
† Number of rats.
[1] P value for normal rats receiving thyroxine compared with normals.
[2] P value for thiouracil-treated rats receiving thyroxine compared with normals.
[3] P value for rats in the cold compared with controls at room temperature.
[I] Injected dose, 4.3×10^7 cts./min. for a 200 gm. rat.

This contrasts to the significant increase in the specific activity of the plasma inorganic P and the significant decreases in the relative specific activities of the adrenal P fractions found in the thiouracil-treated animals not receiving thyroxine (cf. Table 4). However, in rats that were exposed to the cold for 8 days the treatment with thiouracil and thyroxine caused a significant ($P < 0.001$) increase in the specific activity of the plasma inorganic P and significant ($P < 0.05$–0.001) decreases in the relative specific activities of the adrenal P fractions.

In the normal animals exposure to the cold for 8 days caused a significant ($P < 0.05$) decrease in the specific activity of the inorganic P of the plasma and significant ($P < 0.05$–0.01) increases in the relative specific activities of the adrenal fractions as reported by Nicholls and Rossiter (1955). This same effect of 8 days in the cold was evident in normal rats receiving the daily injection of thyroxine. In the animals receiving thiouracil plus thyroxine the cold exposure caused no significant change in the specific activity of the inorganic P of the plasma. The relative specific activities of the adrenal fractions of these animals were increased, although for the adrenal inorganic P the increase did not reach the 5% level of significance. In each instance, the percentage increase brought about by exposure to cold was much less in the group receiving thiouracil plus thyroxine than in the normal animals (12% versus 29% for adrenal inorganic P; 25% versus 35% for 20-minute hydrolyzable P; 18% versus 31% for total acid-soluble P).

DISCUSSION

Leblond and Gross (1943) reported that the adrenal gland is important in the immediate response of a rat to cold and that over a period of days the thyroid gland gradually assumes a role of greater importance. The evidence in favour of the view, expressed by Selyé (1937), that in rats exposed to the cold there are two separate endocrine responses, differing greatly in their time relations, was reviewed by Nicholls and Rossiter (1955), who reported that the two responses were correlated in time with the two phases of increased metabolic activity in the adrenal gland. It was suggested that the biphasic change in the phosphorus metabolism of the adrenal could be interpreted in terms of an increase in the rate at which inorganic P^{32} passes across the cell membrane, i.e. from the extracellular to the cellular fluid.

The Early Phase

The results presented here show that the increase in the phosphorus metabolism of the adrenal gland of the rat exposed to the cold for a short period of time is abolished by removal of the pituitary gland (Table 1). This confirms the observation of Reiss and Halkerston (1950). The finding that short exposures to cold produce changes in the phosphorus metabolism of the adrenal gland similar to those that follow the injection of ACTH

(Gemzell and Samuels, 1950; Riedel *et al.*, 1954), and that the changes produced by exposure to cold are abolished by hypophysectomy, lends strong support to the suggestion that these changes are initiated by stimulation of the cortex of the adrenal by endogenous ACTH released from the pituitary gland.

The above view is further strengthened by the finding that in rats whose thyroid function was depressed with thiouracil, a 3-hour exposure to the cold did not cause a reduction in the adrenal response similar to that observed in hypophysectomized rats (Table 4). These results indicate that the increase in the phosphorus metabolism of the adrenal gland after a short period of cold stress is independent of thyroid function. Despite the decrease in the weight of the adrenals of rats treated with thiquracil (Table 5), such glands are quite sensitive to exogenous ACTH as judged by the ascorbic acid depletion test (Zarrow and Zarrow, 1951; Hess, 1953). In this regard it is of interest to note that the increase in the oxygen consumption, measured either at room temperature (Ring, 1939; MacBeth and Noble, 1949) or in the cold (Ring, 1942), of rats subjected to short periods of cold stress is not affected by removal of the thyroid or treatment with thiouracil.

The Later Phase

The possibility that the later increase in the phosphorus metabolism of the adrenal gland, observed only in rats exposed to the cold for several days, might be secondary to an increase in the activity of the thyroid gland was discussed by Nicholls and Rossiter (1955). Since it is known from both histological (Mills, 1918; Cramer and Ludford, 1926; Kenyon, 1933; Starr and Roskelley, 1940; Bernstein, 1941 and others) and physiological (Dempsey and Astwood, 1943; Leblond *et al.*, 1944; Rand *et al.*, 1952; Catz *et al.*, 1953) studies that there is an increase in thyroid activity in animals exposed to the cold for several days, it seemed reasonable to assume that the stimulation of the metabolism of the adrenal was associated with this increased thyroid activity. The possibility that the adrenal hypertrophy observed during cold stress might be the direct result of increased thyroid activity was discussed by Tepperman, Engel and Long (1943).

The increase in the incorporation of P^{32} into the acid-soluble phosphorus fractions of the adrenal of rats exposed to the cold for a number of days, first reported by Nicholls and Rossiter (1955) and confirmed in these experiments, is the change that would be anticipated if the response of the adrenal to hyperthyroidism were similar to that reported for muscle (Greenberg *et al.*, 1947) or liver (Venkataraman *et al.*, 1950). There is good reason to believe that the adrenal response is, in fact, similar, for thiouracil treatment (Table 4) caused a reduced P^{32} incorporation, which was reversed by thyroxine (Table 6), in animals maintained at room temperature.

The finding that the response in the phosphorus metabolism of the adrenal of animals exposed to the cold for 8 days was greatly decreased when thyroid activity was depressed with thiouracil (Table 6) indicates that part of the later response in the adrenal gland is associated with an increased activity of the thyroid. It should also be noted that the increase in oxygen consumption observed after longer periods of exposure to the cold, in contrast to the findings for shorter periods, is depressed, at least in part, by removal of the thyroid gland or by the administration of thiouracil (Ring, 1939; MacBeth and Noble, 1949).

It seems quite clear from the evidence reported in the literature that there is an increased production of thyroid hormone in rats exposed to the cold for longer periods of time. The studies of Dempsey and Astwood (1943) and Rand *et al.* (1952) indicate that the tissues of animals exposed to the cold possess an increased requirement for thyroid hormone. Treatment with thiouracil causes an increase in the weight of the thyroid, an increase in the mean acinar cell height, a decrease in the serum protein-bound iodine and a decrease in the amount of iodine in the gland. These changes are all reversed by the administration of thyroid hormone, but approximately three times as much hormone is required when the animals are maintained in the cold. A similar increase in the demand for thyroid hormone is shown in the present experiments. The daily injection of 2.5 μg. thyroxine, although sufficient to permit the thiouracil-treated animals to survive 8 days in the cold and to restore to normal the changes in the phosphorus metabolism of the plasma and the adrenal of animals maintained at room temperature, was not sufficient to restore to normal the phosphorus metabolism of the plasma and the adrenals of the rats maintained in the cold (Table 6). In other words, the phosphorus metabolism of the cold-exposed animals demonstrated a requirement for thyroid hormone greatly in excess of 2.5 μg. thyroxine per day, sufficient for animals maintained at room temperature.

SUMMARY AND CONCLUSIONS

In rats exposed to cold stress for 3 hours there was an increase in the incorporation of P^{32} into the acid-soluble phosphorus fractions of the adrenal gland. This increase was abolished by removal of the pituitary. In rats similarly exposed to the cold, but whose thyroid function was depressed by the administration of thiouracil, the phosphorus metabolism of the adrenal was increased to the same degree as in normal animals.

The increase in the phosphorus metabolism of the adrenal observed after 8 days in the cold was still present in the thiouracil-treated rats, but the increase was much smaller than that observed in normal animals.

Evidence is presented for the view that the early (3 hours in the cold) response in the phosphorus metabolism of the adrenal gland of rats exposed to cold is caused by endogenous ACTH liberated from the pituitary.

The later response (several days in the cold) is, in part at least, dependent upon the activity of the thyroid gland.

Acknowledgments

We are grateful to Dr. R. C. Buck for the microscopic examination of the adrenal and thyroid tissue. We thank Mrs. Gertrude Lovegrove for much assistance. One of us (D.N.) is indebted to the National Research Council of Canada for a Studentship.

REFERENCES

ASTWOOD, E. B.: *J. Pharmacol and Exper. Therap.* **78:** 79. 1943.
BAUMANN, E. J. AND D. MARINE: *Endocrinology* **36:** 400. 1945.
BERNSTEIN, J. G.: *Endocrinology* **28:** 985. 1941.
BOATMAN, J. B., J. H. SUNDER, C. RUSS AND C. MOSES: *Proc. Soc. Exper. Biol. & Med.* **78:** 250. 1951.
CATZ, B., I. E. RAWI AND E. GEIGER: *Am. J. Physiol.* **174:** 29. 1953.
COLLIP, J. B. AND L. W. BILLINGSLEY: The Effect of Temperature upon Metabolism, Trans. Amer. Assoc. Goiter. 1936.
CRAMER, W. AND R. J. LUDFORD: *J. Physiol.* **61:** 398. 1926.
DEMPSEY, E. W. AND E. B. ASTWOOD: *Endocrinology* **32:** 509. 1943.
FLOCK, E. V., J. L. BOLLMAN AND J. BERKSON: *Am. J. Physiol.* **155:** 402. 1948.
FREEDMAN, H. H. AND A. S. GORDON: *Proc. Soc. Exper. Biol. & Med.* **75:** 729. 1950.
GEMZELL, C. A. AND L. T. SAMUELS: *Endocrinology* **47:** 48. 1950.
GESCHWIND, I. I., C. H. LI AND H. M. EVANS: *Arch. Biochem.* **31:** 168. 1951.
GREENBERG, D. M., J. FRAENKEL-CONRAT AND M. B. GLENDENING: *Fed. Proc.* **6:** 256. 1947.
HESS, M.: *Proc. Soc. Exper. Biol. & Med.* **84:** 127. 1953.
KENYON, A. T.: *Am. J. Path.* **9:** 347. 1933.
LEBLOND, C. P. AND J. GROSS: *Endocrinology* **33:** 155. 1943.
LEBLOND, C. P., J. GROSS, W. PEACOCK AND R. D. EVANS: *Am. J. Physiol.* **140:** 671. 1944.
LEBLOND, C. P. AND H. E. HOFF: *Endocrinology* **35:** 229. 1944.
MACBETH, R. A. AND R. L. NOBLE: *J. Endocrinol.* **6:** 28. 1949.
MARINE, D. AND E. J. BAUMANN: *Am. J. Physiol.* **144:** 69. 1945.
MILLS, C. A.: *Am. J. Physiol.* **46:** 329. 1918.
NICHOLLS, D. AND R. J. ROSSITER: *Canad. J. Biochem. Physiol.* (In press, May 1955)
PERRY, W. F.: *Canad. J. Med. Sci.* **30:** 36. 1952.
RAND, C. G., D. S. RIGGS AND N. B. TALBOT: *Endocrinology* **51:** 562. 1952.
REISS, M. AND J. M. HALKERSTON: *J. Endocrinol.* **6:** 369. 1950.
RIEDEL, B. E., J. E. LOGAN, H. A. DELUCA AND R. J. ROSSITER: *Canad. J. Biochem. Physiol.* **32:** 251. 1954.
RIEDEL, B. E. AND R. J. ROSSITER: *Canad. J. Biochem. Physiol.* **32:** 261. 1954.
RING, G. C.: *Am. J. Physiol.* **125:** 244. 1939.
RING, G. C.: *Am. J. Physiol.* **137:** 582. 1942.
SELLERS, E. A. AND S. S. YOU: *Am. J. Physiol.* **163:** 81. 1950.
SELYÉ, H.: *Arch. internat. pharm.* **55:** 431. 1937.
STARR, P. AND R. ROSKELLEY: *Am. J. Physiol.* **130:** 549. 1940.
TEPPERMAN, J., F. L. ENGEL AND C. N. H. LONG: *Endocrinology* **32:** 373. 1943.
VENKATARAMAN, P. R., A. VENKATARAMAN, M. P. SCHULMAN AND D. M. GREENBERG: *J. Biol. Chem.* **185:** 175. 1950.
ZARROW, M. X. AND W. L. MONEY: *Endocrinology* **44:** 345. 1949.
ZARROW, M. X. AND I. G. ZARROW: *Proc. Soc. Exper. Biol. & Med.* **76:** 620. 1951.

THE MECHANISM OF DEPRESSION OF PLASMA PROTEIN BOUND IODINE BY 2,4 DINITROPHENOL[1]

R. C. GOLDBERG, J. WOLFF AND R. O. GREEP

Biology Laboratories, Harvard School of Dental Medicine, Boston, Massachusetts

INTRODUCTION

IT HAS previously been shown (Wolff *et al.*, 1950; Goldberg and Chaikoff, 1951) that the administration of 2,4 dinotrophenol (DNP), a potent stimulant of oxygen consumption, results in a prompt and profound fall in the level of plasma protein bound iodine (PBI). A compensatory increase in thyroid stimulating hormone (TSH) output by the anterior pituitary, however, could not be detected. This finding was somewhat surprising in view of the low PBI levels and the "homeostatic" relationship presumed to exist between this level and the anterior pituitary beta cells, the probable source of TSH.

Two fundamental problems were thus presented: 1) by what mechanism did DNP influence the level of PBI and 2) is the presumed "feed-back" relationship between the level of plasma thyroid hormone and the anterior pituitary actually operative?

Since in the earlier studies it was found that with two or three daily injections of DNP neither uptake nor conversion of radioactive iodide to organic form was materially affected, it was felt that thyroid hormone output was not impaired. It was inferred, therefore, that the fall in PBI resulted from either increased peripheral utilization or destruction of the hormone. The data presented here not only bear out this inference but, in addition, suggest that more continuous administration of DNP leads to a diminution in the thyroidal iodide concentrating capacity and a retardation in the rate of release of hormone by the thyroid gland to the circulation. It is felt that the low level of PBI, effected by DNP, can be understood in terms of the alterations in rate of at least three processes involved in iodine metabolism.

EXPERIMENTAL

Sprague-Dawley rats were used in all experiments and were fed either Purina Laboratory Chow or the Remington (1937) low iodine diet (#342 with 2% desiccated liver), as indicated in the various experiments. The iodine content of various lots of Purina Lab

Received for publication November 10, 1954.

[1] This investigation was supported by a grant from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

Chow was found to vary from 1.9 to 3.2 γ/gram; the iodine content of the low iodine diet was found to be approximately 0.1 γ/gram. 2,4-dinitrophenol (DNP) was twice recrystallized from acetone and was added to the diets at a concentration of either 0.15 or 0.2%.

Studies on the I^{131} content of the thyroid, 24 hours after a single intraperitoneal injection (i.p.) of 1–2 μc. I^{131}, were carried out on rats maintained on the low iodine diet for 7–14 days prior to radioiodine administration. At sacrifice the thyroids were weighed, dissolved in 1 N NaOH, diluted, and counted with a thin mica window beta tube.

For the determination of the thyroid-serum I^{131} ratio (T/S ratio) the rats were injected i.p. with 10 mg. propylthiouracil (PTU) in one ml. of an aqueous-ethanolic (7%) solution, pH 9.0. One hour later they were given an i.p. injection of approximately 5 μc. carrier-free I^{131} in 0.5 ml. isotonic saline. One hour after radioiodine administration the rats were anaesthetized with ether, blood was withdrawn by cardiac puncture, and 0.5 ml. serum was plated and counted. The thyroid gland was weighed, dissolved in 0.5 N NaOH, diluted, plated, and counted. The T/S ratio was computed as the ratio of the radioactivity contained in equivalent weights of thyroid gland and serum. In the two experiments (VI and VII) in which the T/S ratio was studied the rats were maintained on Purina Laboratory Chow.

Plasma protein bound iodine (PBI) was determined by the method of Taurog and Chaikoff (1946).

The rate of release of thyroidal organic I^{131} was measured by the method of Wolff (1951). Forty hours after a single intraperitoneal injection of 20 μc. carrier-free I^{131} (at which time more than 90% of the thyroidal I^{131} is in organic form) the thyroidal radioactivity was externally assayed with a bismuth cathode gamma tube; the rats were then injected with 10 mg. PTU and placed on a diet containing 0.1% PTU. At 24-hour intervals thereafter the thyroidal I^{131} was again determined. DNP was added to the diet of the experimental animals at "zero time" (40 hours after I^{131} administration).

The PBI^{131} conversion ratio was determined 24 hours after I^{131} administration as the ratio of the radioactivity contained in the twice washed cold trichloracetic acid (TCA) precipitate of one ml. of serum to the total activity found in one ml. of serum.

The determination of the rate of disappearance of labeled plasma organic iodine was carried out in the following fashion. In the first experiment fifty 100 gm. rats were placed on a diet (Purina Laboratory Chow) containing 0.1% PTU in order to 1) suppress thyroid hormone synthesis and 2) to deplete the animal of organic iodine. Starting 12 days later, all animals were injected subcutaneously each day with 2.0 γ L-thyroxine per 100 grams body weight. On the third day of the thyroxine treatment all rats were injected intraperitoneally with 1 ml. of 0.9% NaCl solution containing 2.5 γL-thyroxine labeled with 6.0 μc. I^{131} (the labeled thyroxine was prepared by exchange and was found to be greater than 95% thyroxine by paper chromatographic analysis). DNP (0.2%) was added to the PTU diets of 25 of the rats one day after the first "stable" thyroxine injection. Groups of rats (5 controls, 5 DNP) were sacrificed at 6, 12, 24, 48, and 72 hours after the labeled thyroxine injection and blood was withdrawn by cardiac puncture. The protein of 1 ml. plasma was precipitated by the addition of 5 ml. cold 10% TCA, and the precipitate was washed three times with 5 ml. portions of cold 5% TCA containing carrier (I^{127}) iodide. The washed precipitate was then dissolved in 1 N NaOH, diluted to a convenient volume and the radioactivity determined with a beta tube. It should be noted that the rats were maintained on PTU for the entire course of the experiment. A second experiment was carried out, identical with the above except that the rats were not injected with thyroxine until zero time when all rats received 2.5 γ L-thyroxine labeled with 8 μc I^{131}. In this case DNP was added to the diets of the experimental animals 4 days prior to thyroxine administration.

RESULTS

The administration of DNP to either male or female rats at a dietary level of 0.15 or 0.2% clearly resulted, in 4 of 5 experiments, in a decrease in thyroidal I^{131} content, as measured 24 hours after a single i.p. injection of carrier-free radioiodide, (Table 1).

Similarly, the administration of DNP for either 10 or 18 days resulted in a depression of the thyroid-serum iodide ratio, one measure of the iodide concentrating capacity of the gland (Experiments VI and VII, Table 1).

In both Experiments IV and V the conversion ratio (plasma PBI^{131}/total plasma I^{131}) was greatly depressed by DNP administration (Table 1).

The fall in plasma PBI (Experiments II, V, VI, Table 1) confirms the depression effected by DNP administration reported earlier (Wolff *et al.*, 1950; Goldberg and Chaikoff, 1951).

TABLE 1. THE EFFECT OF DINITROPHENOL ON THE 24 HOUR UPTAKE OF I^{131} BY THE THYROID GLAND, T/S RATIO, PLASMA PBI, AND CONVERSION RATIO

Exp't.	Treatment*	Period	Thyroid gland I^{131}‡	Plasma PBI^{131} / total I^{131}	Plasma PBI	T/S ratio
		days	%	×100	γ %	
I	0.2% DNP (10)	6	7.2±2.4†			
	Control (9)	6	20.4±4.0			
II	0.15% DNP (10)	6	8.1±1.4		1.3±0.3†	
	Control (10)	6	15.7±2.1		3.6±0.2	
III	0.15% DNP (9)	8	27.0±3.4			
	Control (10)	8	28.1±3.4			
IV	0.2% DNP (7)	6	15.9±2.9	26.1±3.2†		
	Control (7)	6	27.5±2.6	57.9±3.7		
V	0.2% DNP (6)	3	10.2±1.4	39.1±5.3	1.2±0.2	
	Control (6)	3	25.4±3.9	83.2±2.1	3.6±0.2	
VI	0.2% DNP (8)	10			1.4±0.4	15.5±9.2†
	Control (9)	10			3.1±0.2	39.6±8.5
VII	0.2% DNP (6)	18				19.9±11.1
	Control (6)	18				43.5±10.2

* Figure in parentheses represents number of rats/group.
‡ Per cent of injected dose.
† Mean ± Standard Deviation.
Diets: Experiments I–V: Remington low iodine; Experiments VI and VII: Purina Lab Chow.

It is apparent from Table 2 that the addition of DNP to the diet not only greatly interferes with the trapping of iodine by the thyroid, but leads, moreover, to a decrease in the rate at which the iodine, stored within the gland, is released into the circulation. Propylthiouracil, administered to the rats at "zero time," served to prevent any subsequent organification or

TABLE 2. MEAN BIOLOGICAL HALF LIFE OF THYROIDAL I^{131}* AS AFFECTED BY DINITROPHENOL

Experiment	Treatment†	$T_{1/2}$
		days
I	Control (7)	1.2 ±0.3‡
	0.2% DNP (7)	3.0 ±0.4
II	Control (7)	0.9 ±0.2
	0.2% DNP (6)	3.8 ±0.3

* Rats maintained on low iodine diet for 10–14 days prior to I^{131} administration. Organification blocked with propylthiouracil after "zero time" (forty hours after radioiodine injection).
† Figures in parentheses indicate number of rats/group.
‡ Mean ± Standard Deviation.

re-utilization of iodide. Since more than 90% of the radioactive iodine found in the thyroid at zero time is in organic form, the loss of I^{131} furnishes a rough estimate of the rate of release of thyroid hormone to the circulation, even though the chemical nature of all the radioactive iodine leaving the blocked gland has not yet been satisfactorily determined (Goldberg and Wolff, 1954). It can be seen that with DNP the rate of release of iodine into the circulation is slowed to one third to one fourth that of the control rats. It is highly unlikely that such differences can be explained merely by alterations in loss of non-hormonal radioactivity from the gland.

In the study of the rate of disappearance of thyroxine from plasma, PTU-blocked rats were maintained approximately euthyroid with daily subcutaneous injections of thyroxine in the first experiment and were kept hypothyroid in the second. Table 3 clearly reveals that DNP in both cases increased the rate of disappearance of labeled thyroxine from the protein fractions of plasma. The half-time for disappearance was approximately halved by DNP in both experiments. The second experiment was done to rule out the possibility that differences in specific activity of circulating thyroxine might be responsible for a specious difference between the rates of disappearance in the two groups.

TABLE 3. THE EFFECT OF DINITROPHENOL ON THE MEAN HALF TIME FOR THE DISAPPEARANCE OF I^{131}-LABELED THYROXINE FROM PLASMA

Experiment	Treatment	$T_{1/2}$
		hours
I	Control	20
	0.2% DNP	10
II	Control	18
	0.2% DNP	7

Expt. I: PTU fed rats maintained on L-thyroxine.
Expt. II: PTU fed rats not given thyroxine prior to injection of labeled material.

DISCUSSION

The rapid fall in plasma PBI following treatment with DNP could result from three mechanisms, namely: 1) impairment of thyroid hormone synthesis; 2) depression of the rate of release of thyroid hormone into the circulation by the thyroid gland; 3) acceleration of the rate of disappearance of circulating hormone from plasma (by increased utilization or metabolism and/or excretion). The results, presented here, indicate that probably all three mechanisms collaborate in bringing about the fall of the PBI in the DNP-treated rat.

Hormone synthesis depends on at least two processes—iodide uptake and organification. A direct measure of the latter process is not supplied by the data presented here. The uptake, if measured as the 24-hour I^{131} content, is subject to a number of variables (Riggs, 1952). Prominent among these is renal competition for iodide; however, little information is available regarding the effect of DNP in this area. We feel that the depression in 24-hour I^{131} content, resulting from DNP treatment, is probably a significant reflection of a depressed iodide concentrating capacity. This view is strengthened by the data indicating a depression of the T/S ratio, a parameter of thyroid function that is probably independent of variations in renal iodide clearance.

The rate of release of stored thyroidal I^{131} is diminished by DNP treatment; and, as a result of this depression, as well as that in the uptake, a decrease in the conversion ratio (conversion of iodide to circulating PBI) was to be expected. This did, in fact, occur. However, the latter measurement is further influenced by the accelerated breakdown of the PBI (see below) and must hence be interpreted with caution. Increased re-circulation of iodide (I^{131}) would tend to diminish differences in the uptake parameters and was hence disregarded.

It should be clear that uptake, T/S ratios, and release measurements usually reflect variations in thyrotrophic stimulation, and we interpret the above results as due to a diminished TSH stimulation. However, the drop in T/S ratio was not as great as that seen ten days after hypophysectomy, suggesting that TSH output was not completely suppressed.

The demonstration that DNP increases the rate of disappearance of exogenously administered thyroxine from the circulation indicates that still another mechanism is acting to bring about the plasma PBI depression. Not only is hormone being delivered to the circulation at a slower rate, it is also being removed at a faster rate. Two types of experiments were carried out since it initially was believed that DNP was relatively inactive in the hypothyroid rat (Barker, 1946). Hence in the first experiment the rats were maintained relatively euthyroid by daily thyroxine injections. This maneuver, however, added a serious complication, namely the possibility that the specific activity of the circulating thyroxine differed in the two groups. It has been found (Goldberg *et al.*, 1955) that

DNP actually is metabolically quite active in the hypothyroid rat and in the second experiment the complication of an unknown level of stable ("cold") circulating thyroxine was eliminated as none was administered prior to the injection of the labeled hormone. Whether or not the increased rate of disappearance of thyroid hormone reflects increased peripheral metabolism or utilization, or an increase in the rate of excretion is, of course, not revealed in the data presented here.

The finding (Wolff *et al.*, 1950) of a decreased PBI in rats, treated with DNP, suggested that compensatory hyperactivity of the pituitary TSH mechanism might be expected. Such augmentation could not be demonstrated (Goldberg and Chaikoff, 1951) and, indeed, the histological appearance of the thyroid suggested hypoactivity. The present study lends functional confirmation to this surmise and suggests depression of the TSH stimulation of the thyroid by DNP.

Up to the present our understanding of the thyroid-pituitary interrelation has rested on the premise that the thyroid hormone level is the controlling variable. It has never been established, however, as to whether the pituitary responded to the hormone *per se* or to one or more of the effects of the hormone acting locally or elsewhere. To cling to the former concept one must make new assumptions in view of the present findings.

It is possible that the level of plasma PBI bears little relation to the actual level of circulating thyroid hormone or to the thyroid hormone that might influence the beta cells if there is more than one hormone. This possibility has increased somewhat with the demonstration of the existence of triiodothyronine in the plasma of rats and humans (Gross and Pitt-Rivers, 1952; Pitt-Rivers and Stanbury, 1955). There is little doubt that the actual concentration of triiodothyronine in plasma is extremely low if it normally exists there and what one measures in the determination of plasma PBI is for all practical purposes the thyroxine content. Hence, the question of triiodothyronine controling pituitary TSH output must await more refined methods of chemical analysis.

The present study indicates to us that an effect of the thyroid hormone —which can be simulated by DNP—may be the crucial factor in the thyroid-pituitary axis. Other measures which increase oxygen consumption and raise body temperature, such as pyrogenic bacterial extracts 1,2,3,4,-tetrahydro-beta-napthylamine, and elevated ambient temperature, also lead to a depression in the level of plasma PBI (Goldberg, 1954; Goldberg *et al.*, 1955). The fact that under some of these conditions there is an apparent decrease in stimulation of the thyroid by TSH would lend weight to the concept that the level of plasma PBI is not the stimulus or inhibitor *per se* to the pituitary beta cells or, at least, is not the only influence. One must therefore consider the possibility that it is not (only) the actual level of circulating thyroid hormone(s) that regulates pituitary TSH output but some aspect(s) of its peripheral function(s) acting directly on the pituitary or indirectly through the central nervous system.

SUMMARY

The marked fall in plasma protein bound iodine (PBI) effected by DNP is confirmed.

DNP administration results in a decrease in 1) the thyroidal 24-hour uptake of I^{131}; 2) the thyroidal iodide concentrating capacity and 3) the ratio of plasma PBI^{131} to total plasma I^{131} (conversion ratio) 24 hours following I^{131} administration.

The rate of release of thyroidal organic iodine into the circulation is decreased and the rate of disappearance of thyroid hormone from the circulation is increased by DNP administration.

It is felt that the rapid fall in plasma PBI effected by DNP can be readily understood in terms of these rate changes.

The relationship of the actual level of circulating thyroid hormone to the output of thyroid stimulating hormone (TSH) by the anterior pituitary is discussed; it is suggested that factors other than the plasma PBI influence TSH output by the pituitary beta cells.

REFERENCES

BARKER, S. B.: *Endocrimology* **39**: 284. 1946.
GOLDBERG, R. C.: *Fed. Proc.* **13**: 184. 1954.
GOLDBERG, R. C. AND I. L. CHAIKOFF: *Endocrinology* **49**: 613. 1951.
GOLDBERG, R. C. AND J. WOLFF: *Endocrinology* **54**: 181. 1954.
GOLDBERG, R. C., J. WOLFF AND R. O. GREEP: In Preparation. 1955.
GROSS, J. AND R. PITT-RIVERS: *Lancet* I: 439. 1952.
PITT-RIVERS, R. AND J. B. STANBURY: *J. Clin. Endocrinol. and Metab.* 1955.
REMINGTON, J.: *J. Nutr.* **13**: 223. 1937.
RIGGS, D. S.: *Pharmacol. Rev.* **4**: 284. 1952.
TAUROG, A. AND I. L. CHAIKOFF: *J. Biol. Chem.* **163**: 313. 1946.
WOLFF, J.: *Endocrinology* **48**: 284. 1951.
WOLFF, J., L. RUBIN AND I. L. CHAIKOFF: *J. Pharmacol. and Exp. Therap.* **98**: 45. 1950.

INTERRELATIONSHIPS OF ANTERIOR PITUITARY AND ADRENAL CORTEX IN THE RAT OVARIAN HYPEREMIA REACTION[1]

ELIZABETH KNAPP SMITH

Department of Obstetrics and Gynecology, University of Washington, Seattle

THE ovarian hyperemia response to chorionic gonadotropin in the immature rat is sufficiently reproducible to be widely used as an accurate, standard test for pregnancy. Albert (1951) has made the response the basis of a quantitative assay for chorionic gonadotropin. However, the function of the response in ovarian physiology is not well understood.

In investigating the interrelationships between ovary and adrenal cortex, and of the effects of adrenal cortical hormones upon the response of the ovary to gonadotropin, it was noted that cortisone interfered with the hyperemia response to chorionic gonadotropin. This finding suggested the possibility that the wide-spread use of cortisone as a therapeutic agent might render the "Rapid Rat" test for pregnancy unreliable under certain conditions. Soon after inaugurating the studies, Payne (1951) published a report indicating that the adrenal cortex or its hormone(s) is essential for the ovarian hyperemia response to chorionic gonadotropin. More recently, DeCosta (1953) has shown that cortisone does not interfere with the action of exogenous gonadotropin in inducing ovulation in the rabbit. These variations in the effect of cortisone emphasize the need for further study of the relationship between the adrenal cortex and the ovary.

EXPERIMENTAL

The ovarian hyperemia test was carried out according to the method outlined by Albert (1951). Weanling female rats of the Wistar strain, weighing 35–50 gm., were used for most of the experiments. However, since the hypophysectomized animals available to us were of the Long-Evans strain, a few tests were performed using intact Long-Evans rats. Entirely similar results were obtained with both species. Animals were hypophysectomized at 21–22 days of age, shipped from Berkeley by air express after a 24 hour recovery period, injected with cortisone or other hormones on the 3rd post-operative day, and tested for the hyperemia response to chorionic gonadotropin on the 4th day after operation. Adrenalectomy was performed using the bilateral dorsal approach and the animals were given physiological saline for drinking water postoperatively. Hormone injections were begun on the first or second day after operation and the response to chorionic gonadotropin was determined on the second or third postoperative day.

Received November 13, 1954.

[1] This investigation was in part supported by State of Washington Initiative 171 funds for research in biology and medicine.

The standard human chorionic gonadotropin preparation[2] was given intraperitoneally in dilution with saline. Dilutions were adjusted to a constant injection volume of 2 ml., which was administered 1 ml. on either side of the abdomen. Dilutions of the chorionic gonadotropin had to be freshly prepared just prior to use, or could be stored in the deep freeze if thawed and refrozen no more than twice.

Corticotropin,[3] cortisone[4] and other steroid compounds were injected intramuscularly on the inner aspect of the hind limb of the rat. Alternate limbs were used for repeated injections. Usually injections were made morning and afternoon of the day prior to chorionic gonadotropin administration, and again immediately before the gonadotropin injection. Thus cortisone and hydrocortisone[5] were given 3 times within 24 hours prior to chorionic gonadotropin; desoxycorticosterone in oil and corticotropin were given only twice, once on the day before and once on the day of the chorionic gonadotropin injections.

Four hours after the injection of chorionic gonadotropin, the animals were killed with ether and the color of the ovaries judged under standard conditions. Throughout the study color observations were made by the same observer, with the same daylight surgical lamp at constant height above the table, and using the same background paper. These precautions were made in an attempt to avoid as much as possible the subjective difficulties inevitably encountered in using a visual color endpoint.

The ovaries were classified as positive or negative as they would be judged in the interpretation of a pregnancy test. Doubtful reactions, (both ovaries doubtful or one ovary positive and one ovary negative), were classified as negative. A positive response in one ovary with a doubtful response in the other was considered positive; a negative response in one ovary with a doubtful response in the other was considered negative. Controls using intact uninjected and gonadotropin-injected animals were included in each experiment. Results reported below represent the sum of several experiments in which similar results were obtained in different groups of animals.

RESULTS

Dosage-response curves established the maximal response, approximately 95% of animals tested giving a positive response, with 4 I.U. of either preparation of chorionic gonadotropin. This dosage level was then used for the majority of the subsequent studies.

As shown in Table 1, cortisone acetate in dosage of 2.5 mg. caused a significant inhibition of the ovarian hyperemia response to chorionic gonadotropin in intact rats. The percentage of positive responses was markedly reduced at all levels of chorionic gonadotropin administration. Significant inhibition of the response also occurred with lower dosages of cortisone; i.e., 1.25 mg. cortisone at 3 different levels of chorionic gonadotropin administration reduced the percentage of positive responses to values ranging

---

[2] USP Chorionic Gonadotropin; and "Pranturon" generously supplied by Schering Corporation, Bloomfield, New Jersey.

[3] ACTHAR Gel kindly supplied by Dr. Irby Bunding of Armour Laboratories, Chicago.

[4] Cortisone Acetate, CORTONE, suspension, kindly supplied by Dr. E. Alpert, Merck and Company, Rahway, New Jersey.

[5] Hydrocortisone, CORTRIL, kindly supplied by Dr. Harry Rudel, Charles Pfizer and Company, Inc., New York.

TABLE 1. EFFECT OF ADRENAL STEROIDS AND CORTICOTROPIN ON OVARIAN HYPEREMIA IN INTACT RATS

Ovarian response	Number of animals						Per cent*
	Total	−−	±−	+−	±+	++	+
Controls:							
No injection	22	19		2	1	0	4.5
Saline	5	5				0	0
Corticotropin	7	7				0	0
Cortisone	16	16				0	0
Desoxycorticosterone	2	2				0	0
Hydrocortisone	2	1	1			0	0
Chorionic Gonadotropin:							
0.5 I.U.	5	1			2	2	80
1.0 I.U.	5		3		1	1	40
1.5 I.U.	17	2	1	2		12	70.5
2.0 I.U.	22			1	1	20	95.5
3.0 I.U.	20	2		3	1	14	75
4.0 I.U.	68			3	2	63	95.6
CG+Cortisone (acetate)							
1.5 I.U.+2.5 mg.×3	16	6	1	4	2	3	31.2
2.0 I.U.+2.5 mg.×3	37	8	2	11	6	10	43.3
3.0 I.U.+2.5 mg.×3	15	6		1	2	6	46.6
4.0 I.U.+2.5 mg.×3	33	11	6	3	7	6	39.4
2.0 I.U.+1.25 mg.×3	10	6	1	1		2	20
3.0 I.U.+1.25 mg.×3	15	1	1	4	1	8	60
4.0 I.U.+1.25 mg.×3	24	5	3	5	2	9	58.3
4.0 I.U.+0.1 mg.×3	18	1		3	3	11	77.8
CG+Hydrocortisone (free alcohol)							
4.0 I.U.+1.0 mg.×3	6	3	1	1		1	16.7
4.0 I.U.+0.5 mg.×3	10	1	1	2	4	2	60
4.0 I.U.+0.1 mg.×3	10			2	2	6	80
CG+Desoxycorticosterone acetate							
4.0 I.U.+0.5 mg.×2	10	0				10	100
CG+Corticotropin (ACTHAR GEL)							
4.0 I.U.+6 I.U.×2	23	1	1	1	3	17	87
4.0 I.U.+3 I.U.×2	16			1	1	14	93.8
4.0 I.U.+1 I.U.×2	6					6	100

* Including one ovary +, other ±.

from 20–60%. Figure 1 illustrates that with a standard amount of chorionic gonadotropin, 4 I.U., a progressive decrease in percentage of positive responses was observed as the dosage of cortisone was increased. The sharp change in the slope of the dosage-response curve between levels of 1.25 and 2.50 mg. cortisone may indicate secondary effects of the higher dosage of cortisone upon protein metabolism and other body processes. The lowest dosage of 0.1 mg. cortisone represents approximately 2.5 mg./kg., and corresponds to a dose of 150 mg. for a 60 kg. woman.

The data in Table 1 also indicate that hydrocortisone blocks the response of the ovary to chorionic gonadotropin in the rat. Although the number of animals is small, a similar relationship between degree of interference with the hyperemia response and the amount of hydrocortisone administered is apparent with hydrocortisone, as with cortisone. Corticotropin had essen-

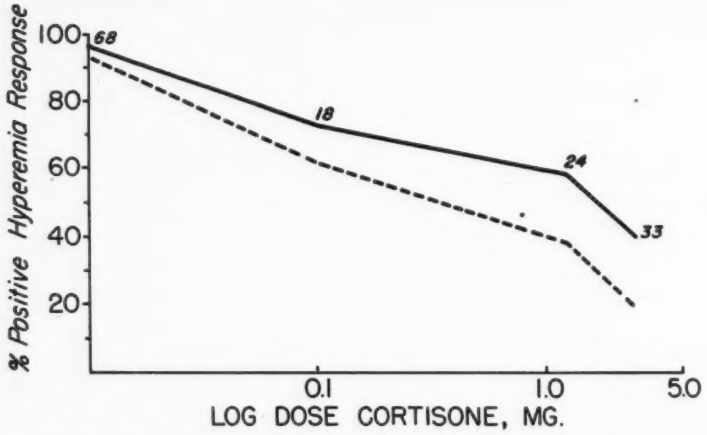


FIG. 1. Effect of increasing cortisone dosage upon percentage of positive hyperemia responses to a standard dosage of 4 I.U. chorionic gonadotropin. Number of animals at each dosage level is noted. Broken line: values when both ovaries were positive; straight line: values when results in which one ovary was positive and one doubtful are included.

tially no inhibitory effect upon the ovarian hyperemia response in intact animals. Desoxycorticosterone acetate in the high dosage of 0.5 mg. also had no effect upon the hyperemia response in the 10 rats tested.

Early in the study it was realized that figures representing only the number of positive responses did not give an accurate picture of the effect of cortisone, because of the large number of mixed doubtful and positive responses and the noticeably decreased intensity of positive responses. In an effort to make the results more quantitative and therefore more meaningful, the color of the ovary was estimated using the Munsell color chart, Red (5.0R), recommended by Farris (1946) for his studies of ovulation. According to the Munsell system any color can be described accurately by a fraction in which the numerator denotes "value" and the denominator, "chroma." The smaller the number in the numerator, the deeper the color; and the larger the number in the denominator, the brighter the color. After noting whether the test was positive, negative, or doubtful, on the basis of the pregnancy test interpretation, each ovary was compared to the color chart and a numerical fraction describing the color was recorded.

The effects of the cortisone injections on hyperemia, based on the Munsell color readings, are presented in Figure 2. In this figure, there is a gradual progression from deep red to almost colorless as the numbers on the abscissa change from 4/8 to 8/4. The reading of 4/8 is strongly positive while readings of 8/4 and 7/4 are negative. Readings of 7/8 and 7/6 usually were obtained in ovaries judged to be doubtful or negative. Ovaries giving readings deeper than 7/8 were almost always positive. Because the two ovaries in one animal frequently differed in depth of color response, the

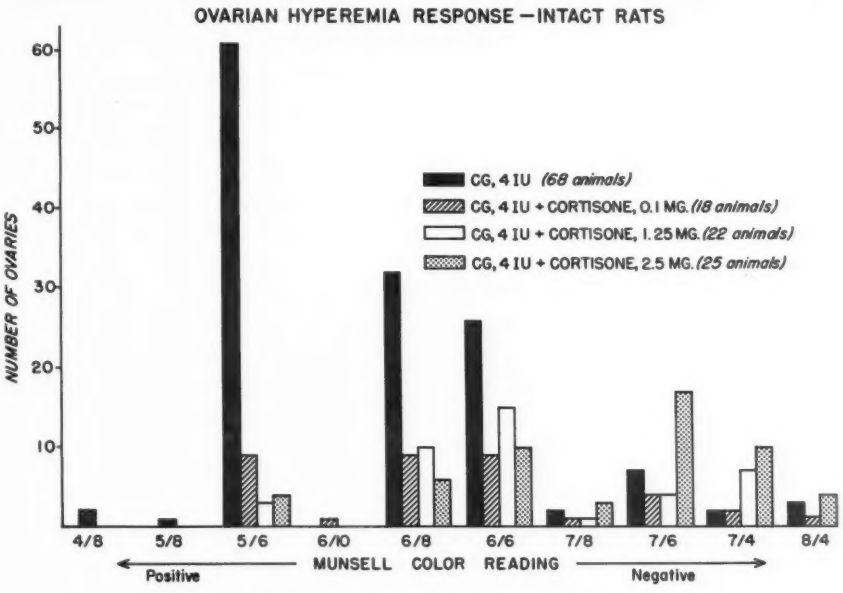


FIG. 2. Effect of increasing cortisone dosage upon depth of color in hyperemia response of ovary to chorionic gonadotropin.

reading for each ovary is plotted separately. It is obvious that cortisone at either of the 3 dosage levels causes a distinct depression in the intensity of the hyperemia compared to the control animals. Fewer strongly positive (deep red) responses and more intermediate and negative responses occur when cortisone is administered. Again, it would appear that the higher the dosage of cortisone, the greater the depression of the hyperemia response.

Table 2 shows that hypophysectomy prevented the response to chorionic gonadotropin in approximately 55% of the animals tested, with only 45% giving a positive response. Corticotropin restored the response in 90% positive; but cortisone and desoxycorticosterone in the dosages used had no significant effect upon the percentage of positive responses.

The data in Table 3 indicated that adrenalectomy also decreased the percentage of positive responses to only 39%. Neither corticotropin nor desoxycorticosterone had any significant effect upon the response. Cortisone, however, increased the percentage of positive responses; the dosage of 1.25 mg. increasing this figure to 71%. Likewise the higher dosage of hydrocortisone, 1.0 mg., significantly increased the responses to 62.5% positive. The fact that the smaller amounts of cortisone and hydrocortisone were not effective suggests the existence of a threshold level for adrenal cortical hormones in their influence upon the response of the ovary to gonadotropin.

TABLE 2. EFFECT OF ADRENAL STEROIDS AND CORTICOTROPIN ON OVARIAN HYPEREMIA IN HYPOPHYSECTOMIZED RATS

Ovarian response	Number of animals						Per cent*
	Total	−−	±−	+−	±+	++	+
Control	6	6				0	
Chorionic gonadotropin (CG), 4 I.U.	22	6	3	3	2	8	45.5
ACTH 6 I.U.	5	5				0	0
CG, 4 I.U.+ACTH 6 I.U.	10	1				9	90
Cortisone 2.5 mg.	5	5				0	0
CG 4 I.U.+Cortisone 2.5 mg.	10	2	2	1	1	4	50
CG 4 I.U.+Cortisone 1.25 mg.	16	5	2	3	1	5	37.5
CG 4 I.U.+Cortisone 0.1 mg.	16	9	2		2	3	31.2
CG 4 I.U.+Hydrocortisone 0.5 mg.	6	4	1		1	0	0
CG 4 I.U.+Hydrocortisone 0.1 mg.	5	1	4			0	0
DOCA 2.5 mg.	2	2				0	0
CG 4 I.U.+DOCA 2.5 mg.	8	3	1	1	2	1	37.5
CG 0.2 I.U.	4	2	1	1		0	0
CG 0.2 I.U.+Cortisone 1.25 mg.	4	4				0	0

* Including one ovary + other ±.

DISCUSSION

Explanation of the observed results is difficult. However, it is recognized that some pituitary gonadotropin must be present in order for chorionic gonadotropin to stimulate function of the immature rat ovary (Zondek 1946), and it has been demonstrated that the use of the ovarian hyperemia response as a test for pregnancy may be improved by the addition of a "pituitary synergist" (Fried 1950). Presumably, in order to prevent the response one need only block the minute amounts of endogenous gonadotropin circulating in the immature female rat.

A plausible explanation for our results would be that cortisone in large dosage, which is known to inhibit the release of corticotropin, may also inhibit the release of gonadotropin to such an extent that insufficient pituitary gonadotropin is available for the ovarian hyperemia response to occur. In the intact rabbit, in which the necessity for endogenous pituitary go-

TABLE 3. EFFECT OF ADRENAL STEROIDS AND CORTICOTROPIN ON OVARIAN HYPEREMIA IN ADRENALECTOMIZED RATS

Ovarian response	Number of animals						Per cent*
	Total	−−	±−	+−	±+	++	+
Control	7	7				0	
Chorionic gonadotropin (CG) 4 I.U.	34	8	8	5	4	9	38.9
CG 4 I.U.+ACTH 3 I.U.	6	6				0	0
Cortisone 2.5 mg.	7	7				0	0
CG 4 I.U.+Cortisone 2.5 mg.	20	4	2	3	3	8	55
CG 4 I.U.+Cortisone 1.25 mg.	28	6	1	1	4	16	71.5
CG 4 I.U.+Cortisone 0.1 mg.	16	6	1	2	1	6	43.7
CG 4 I.U.+DOCA 1.5 mg.	12	1	3	3	1	4	41.5
Hydrocortisone 0.5 mg.	1					0	
CG 4 I.U.+Hydrocortisone 1.0 mg.	8	1	1	1	2	3	62.5
CG 4 I.U.+Hydrocortisone 0.5 mg.	8	1	1	2	2	2	50

* Including one ovary + other ±.

nadotropin has not yet been demonstrated and may not be required, cortisone did not interfere with the induction of ovulation by chorionic gonadotropin injection (3).

Hypophysectomy also removed pituitary gonadotropin and therefore prevents the hyperemia response. Ovarian hyperemia was restored by injections of corticotropin in the hypophysectomized rats. Perhaps the preparations of corticotropin used contained sufficient traces of gonadotropin to permit the reaction to occur.

The failure of response to chorionic gonadotropin in adrenalectomized animals may have another explanation. As suggested by Payne (1951), the adrenal cortex and/or its hormones may be necessary for the response, acting in a "permissive" fashion as outlined for adrenocortical hormones by Ingle (1952), and others. The finding that cortisone and hydrocortisone at least partially restored the hyperemia response to chorionic gonadotropin in adrenalectomized animals (Table 3) confirms the work of Payne and of Behrman (1954).

In the rat it has been found (Bush, 1953) that adrenal vein blood contains primarily corticosterone instead of hydrocortisone and that corticosterone is the principal steroid produced under corticotropin stimulation (Morris, 1953). Assuming corticosterone is less effective than hydrocortisone in influencing the hyperemia response, these observations may explain the lack of effect of corticotropin in the intact rats, even in the excessive amounts used. However, in the hypophysectomized animals, perhaps corticotropin caused release of enough hydrocortisone along with corticosterone to permit the response to occur.

The amounts of cortisone necessary to inhibit the hyperemia response are so high that the likelihood of any interference by cortisone therapy in the use of this response as a pregnancy test is very remote. Values for urinary 17-hydroxycorticoids seldom exceed 10 mg./24 hours, even with moderate cortisone therapy; therefore, there is little possibility of reaching a concentration of as much as 1 mg. of cortisone in the 2 ml. of urine usually injected into a rat for the pregnancy test.

Hydrocortisone and cortisone have similar effects in all 3 groups of animals. However, hydrocortisone appears to be approximately twice as effective as cortisone, since a similar degree of inhibition of the hyperemia response occurred with 1.0 and 0.5 mg. hydrocortisone as with 2.5 and 1.25 mg. of cortisone acetate (Table 1).

SUMMARY

Cortisone and hydrocortisone were found to interfere significantly with the ovarian hyperemia response to human chorionic gonadotropin in the intact immature rat. The interference was demonstrated by a decrease both in percentage of positive responses and in degree of hyperemia as ex-

pressed by depth of color on the Munsell color chart. Hypophysectomy and adrenalectomy also interfered with the hyperemia response. Corticotropin restored this effect in hypophysectomized rats. Cortisone and hydrocortisone were partially effective in restoring the response in adrenalectomized animals. A tentative explanation for these results is presented.

REFERENCES

ALBERT, A. AND J. BERKSON: *J. Clin. Endocrinol.* **8**: 805. 1951.

BEHRMAN, J.: Personal communication.

BUSH, I. E.: Ciba Foundation Colloquia on Endocrinology. Vol. VII, p. 210, Little, Brown & Co., Boston, 1953.

DECOSTA, E. J. AND M. A. ABELMAN: *Obstetrics and Gynecology* **1**: 269. 1953.

FARRIS, E. J.: *Am. J. Ob. & Gyn.* **52**: 14. 1946.

FRIED, P. H. AND A. E. RAKOFF: *J. Clin. Endocrinol.* **10**: 423. 1950.

INGLE, D. J.: *J. Endocrinol.* **8**: xxiii. 1952.

MORRIS, C. J. O. R. AND D. C. WILLIAMS: *Ciba Foundation Colloquia on Endocrinology*, Vol. VII, p. 261, Little, Brown, & Co., Boston, 1953.

PAYNE, R. W.: *Proc. Soc. Exper. Biol. Med.* **77**: 242. 1951.

ZONDEK, B. AND F. SULMAN̆: *Vitamins and Hormones.* Vol. III, p. 328, The Academic Press, Inc., New York, 1946.

EFFECTS OF GONADAL HORMONES ON BLOOD SUGAR OF THE CHICKEN[1]

PAUL D. STURKIE

Laboratory of Avian Physiology, Rutgers University, New Brunswick, New Jersey

THE published values for blood sugar of chickens show considerable variation (see Shimer 1937; Sturkie, 1954). Sex differences have received little attention. Most of the determinations have been made on the female, and in some instances, sex was not indicated. Values for adult males (Burrows *et al.*, 1935) were of the same magnitude, or higher, than those reported for females by others (Murray and Rosenberg, 1953; Shimer, 1937). It is likely that much of the variation results from differences in methods.

The results of Rogemont (1930a & b), based on small numbers, indicate that blood glucose of laying hens is 30 per cent higher than in adult males, but the levels in sexually immature males and females are approximately the same. The sugar level decreased in males at sexual maturity, suggesting the depressing effect of androgen. The level in the castrate male was of the same order as in laying females. More recent studies by Hanke (1953) also showed that blood sugar decreases in males at sexual maturity.

PROCEDURE

Blood glucose was determined by the ferricyanide-method of Kingsley and Reinhold (1949) except that dilute tungstic acid was used to precipitate the plasma proteins from 0.1 ml. of blood instead of acid cadmium sulfate. Various dilutions of the latter were tried on chicken blood, but they produced values that were much too low. Because of the appreciable amounts of non-glucose reducing substances in mammalian blood, values obtained when tunstic acid is used are much too high. According to Cavett (1939) the non-glucose reducing substances in chicken blood are much lower, and therefore account for about 6 per cent of color reduction when tungstic acid is used.

Most of the sugar determinations were made in the morning of the same day on an equal number of birds in each category. However, a few determinations were made on blood samples drawn in the afternoon, after it had been demonstrated that this did not influence the values.

RESULTS

Blood Sugars of Males, Females and Capons

Mean blood sugars for fed and starved birds are presented in Table 1. The level for fed females is considerably higher than for males, and slightly

Received November 15, 1954.

[1] Paper of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, Department of Poultry Science.

TABLE 1. BLOOD SUGAR VALUES OF NORMAL MALES, FEMALES AND CAPONS, FED (F), AND STARVED, 17–24 HOURS (S)

	Males		Females		Capons	
	1	2	3	4	5	6
	S	F	S	F	S	F
No.	17†	33‡	18†	56‡	12	44†
Mean*	172.4	197.8	181.5	226.0	180.0	215.2
S.E.	2.6	2.3	3.1	2.8	4.4	2.4

* All mean differences between groups statistically significant except between 3 and 5.
† Represents summary of 2 different groups.
‡ Represents summary of 3 different groups.

but significantly higher than that of capons. The level for capons is also higher than that of males. Fasting for 17 to 24 hours reduced the blood sugar level significantly in each of the three groups, but the degree of depression (20%) was higher in the female. Consequently, the fasted levels of capons and females were not appreciably different.

Effects of Androgen on Blood Sugar

The suggestion that androgen depresses blood sugar was tested by injecting females and capons with 5 mg. each of testosterone propionate every other day for 14 days. During this period there was a considerable increase in comb size. The results are presented in Table 2. Androgen depressed blood sugar levels in females and capons significantly, but the levels were still higher than that of the normal male.

Effects of Estrogen on Blood Sugar

Diethylstilbestrol was administered to adult males and capons at a level known to suppress output of pituitary gonadotrophins. Each bird was implanted with three, 15-mg. pellets over a 4 week period. Comb size was markedly reduced in males during this period, an indication of depressed

TABLE 2. EFFECTS OF ANDROGEN ON MEAN BLOOD SUGAR OF CAPONS AND FEMALES (NON-STARVED)

	Females		Capons	
	A	B	C	D
	Control	Androgen	Control	Androgen
No.	12	12	12	12
Mean	233.5	214.6	222.9	207.0
S.E.	3.2	4.8	4.3	5.8

Mean Differences: A & B – P= <0.01
C & D – P = >0.04

TABLE 3. EFFECTS OF ESTROGEN ON MEAN BLOOD SUGARS OF MALES AND CAPONS (NON-STARVED)

	Males		Capons	
	A	B	C	D
	Control	Estrogen	Control	Estrogen
No.	21	20	20	21
Mean	193.0	196.3	213.0	194.4
S.E.	3.12	4.51	3.55	2.49

Mean Differences: A & B – P = >0.05
C & D – P = <0.01

androgen secretion by the testes. Two groups of birds at different times of the year received the treatment. The results were the same in both groups, hence the data are grouped (Table 3). Estrogen depressed blood sugar significantly in the capons but had no appreciable effect in males.

DISCUSSION

These results show that the blood sugar of non-starved adult males is approximately 14% lower than in females, instead of 30% as reported by Rogemont (1930a). Moreover, the level for capons, even though considerably higher than for males, is significantly lower than for females. The data indicate that androgen production by the male accounts for most of the difference in blood sugar level of the sexes, and the results obtained with androgen support it. Hanke (1953) who reported that the cock is more sensitive to insulin shock than the hen believed that sex hormones were responsible for the difference. The fact that following castration in the male, blood sugar level does not return completely to the female level indicates that factors other than androgen are also involved in the regulation of blood sugar.

The results obtained with estrogen were unexpected. Suppression of androgen output should have elevated blood sugar in the estrogenized male, unless estrogen has a directly depressing effect on blood sugar, which in this case counteracted the rise anticipated after androgen inhibition. The fact that estrogen depressed blood sugar in the capon suggests that it does have such an effect, at least when administered in unphysiological doses. The effects of large, and physiological doses of estrogen on the normal and castrated female remain to be determined. Estrogen reduces food intake and body weight in mammals. The same effect on chickens might account for the depressing effect on blood sugar. Estrogen, however, at the dosages used in these experiments does not depress feed consumption and body weight, but may actually increase body weight, and sometimes food intake (Lorenz, 1945a & b; Jaap & Thayer, 1944: see review of Sturkie, 1954). Diethylstilbestrol is used commercially by poultrymen to improve

market quality of poultry. Food intake was not determined in these experiments, but body weights were recorded. The gains for the estrogenized males and capons averaged 409 and 546 gm., and for the control males and capons 228 and 273 gm. respectively.

The data of Riddle (1937) suggest that the production of estrogen coincident with ovulation increases blood sugar of the pigeon. The administration of estrogen to ducks did not influence the blood glucose level, according to Mirsky *et al.* (1941). The effects of sex hormones on blood sugar of certain mammals have been highly variable and inconclusive (Dolin *et al.*, 1941).

SUMMARY

Blood sugar determinations were made on fed and starved males, females and capons, and on some of these after administration of androgen and estrogen.

The blood sugar level for fed females is appreciably higher (14%) than that of males, and slightly but significantly higher than for capons. The level for capons is also significantly higher than that for males.

Fasting for 17 to 24 hours reduced the sugar level significantly in each of the three groups, but the degree of depression (20%) was higher in females. The fasted sugar levels of capons and females did not differ appreciably.

Testosterone propionate injected every other day for 14 days (35 mg.) into females and capons depressed blood sugar significantly, but the levels were still higher than that of the male.

Diethylstilbestrol pellets implanted, and effective over a four-week period reduced blood sugar significantly in capons, but had no effect in males.

REFERENCES

Burrows, W. H., J. C. Fritz and W. H. Titus: *J. Biol. Chem.* **110:** 39. 1935.
Cavett, J. W.: *Poultry Sci.* **32:** 805. 1941.
Dolin, G., S. Joseph and R. Gaunt: *Endocrinology* **28:** 840. 1941.
Hanke, O.: *Z. ges. exp. Med.* **122:** 238. 1953.
Jaap, R. G. and R. H. Thayer: *Poultry Sci.* **23:** 249. 1944.
Kingsley, G. R. and J. G. Reinhold: *J. Lab. and Clin. Med.* **34:** 713. 1949.
Lorenz, F.: *Poultry Sci.* **24:** 128. 1945a.
Lorenz, F.: *Poultry Sci.* **24:** 91. 1945b.
Mirsky, I. H., N. Nelson, I. Grayman and M. Korenberg: *Am. J. Physiol.* **135:** 223. 1941.
Murray, H. C. and M. M. Rosenberg: *Poultry Sci.* **32:** 805. 1953.
Riddle, O.: Cold Spring Harbor Symposia on Quantitative Biol. **5:** 362. 1937.
Rogemont, L.: *Comp. Rend. Soc. Biol.* **104:** 154. 1930a.
Rogemont, L.: *Comp. Rend. Soc. Biol.* **104:** 256. 1930b.
Shimer, S. R.: *Univ. New Hampshire Tech. Bull.* **69:** 1937.
Sturkie, P. D.: Avian Physiology. Comstock Pub. Co., Ithaca, New York, 1954.

THE EFFECT OF GROWTH HORMONE ON LIPOGENESIS IN INTACT AND ADRENALECTOMIZED RATS

WILLIAM F. PERRY AND HELEN F. BOWEN

Department of Physiology and Medical Research, The University of Manitoba, Winnipeg, Canada

GROWTH hormone has been shown to affect both fatty acid formation (Welt and Wilhelmi, 1950; Brady, Lukens and Gurin, 1951) and oxidation (Greenbaum and McLean, 1953) but the significance of its role in these processes is not clear. Welt and Wilhelmi (1950) showed that growth hormone depressed the incorporation of deuterium labelled water into carcass and liver fats of rats. Brady, Lukens and Gurin (1951) noted that hypophysectomy brought about an acceleration of fat formation from carbohydrate. These workers also demonstrated that the treatment of 'Houssay' cats with growth hormone inhibited lipogenesis. Greenbaum and McLean (1953) found that growth hormone mobilized fat in the liver and caused an increase in liver fatty acid oxidase.

From findings of this sort it is generally considered that growth hormone has an inhibitory action on fat formation but apart from the demonstration by Welt and Wilhelmi (1950) that growth hormone inhibited the incorporation of deuterium into body and liver fats there has been little investigation of the effect of growth hormone on fat formation in the intact animal.

The actual part played by the adrenal cortex in the overall metabolism of fat is not entirely clear. Adrenal steroids have been shown to inhibit lipogenesis (Balmain *et al.*, 1954; Brady *et al.*, 1951) and adrenalectomy has been reported to bring about an increase in the incorporation of D_2O into carcass and liver fats (Welt and Wilhelmi, 1950). Apart from this there appear to have been very few observations on the process of lipogenesis in the adrenalectomized animal alone although it has recently been demonstrated that the incorporation of acetate into fatty acids by liver slices is depressed in the adrenalectomized rat (Perry and Bowen, 1955).

It was thought of interest to re-examine the action of growth hormone in fat metabolism by observing the effects on lipogenesis of the administration of growth hormone to the intact animal. Since both growth hormone and adrenal cortical hormones appear to be concerned with fat metabolism it was of interest to determine whether they act independent of one another or are related in this respect i.e. to ascertain whether growth hormone

Received November 19, 1954.

affects fat formation not only in the intact animal but also in the absence of the adrenal cortex.

METHODS

The experimental animals were male albino rats 3½ months old weighing 180–220 gm. The adrenalectomized animals were used 7–9 days after adrenalectomy. Following the operation they were placed on a Fox Chow diet with 1% NaCl as drinking water.

As nutritional status has been shown to markedly affect lipogenesis the experiments were done on two series of animals. Both series consisted of four groups of animals: intact rats, intact rats given growth hormone, adrenalectomized rats and, adrenalectomized rats given growth hormone. The first series consisted of animals allowed free access to food (Fox Chow) at all times; the second series was made up of "Force-Fed" animals. These were fed also on Fox Chow, the adrenalectomized animals eating it *ad libidum;* each operated rat had an intact control which was pair fed with his adrenalectomized partner. Twenty hours before sacrifice all animals of this group were given 3.5 gm. glucose by stomach tube and this dose of glucose was repeated again four hours before killing. In the experiments with growth hormone, it was freshly dissolved in normal saline at pH 10.0 and injected intraperitoneally in 5 mg. doses 3½ hours before sacrifice.[1]

The animals were killed by decapitation, the livers removed and slices prepared with a Stadie-Riggs microtome. 1100–1500 mg. slices were taken and placed in 10 ml. Krebs-Ringer bicarbonate to which was added 2 microcuries of 2-C^{14} sodium acetate. The final acetate concentration in the medium was 1.5 micromole per ml. The flasks were shaken at 37° C. for 3 hours in an atmosphere of 95% 0_2 and 5% CO_2.

At the end of this time the flask contents were hydrolysed, and the fatty acids isolated, plated and counted (Felts *et al.*, 1951) and the fraction of the acetate converted into liver fatty acid calculated.

RESULTS

During the 7–9 day period following adrenalectomy the food intake of animals allowed free access to food at all times, was quite constant being 15 ± 1 gm. daily of the Fox Chow. With food intake at this level the body weight changed little; none gained weight while a few lost weight amounting to 10 gm. at the most. This weight loss in these few animals took place during the first 2–3 days postoperative; thereafter weight remained unchanged.

In the group of intact animals with food intake restricted to the level of the adrenalectomized rats the body weight remained unchanged.

The group of intact animals that was allowed free access to food at all times presumably ate more than 15 gm. per rat of chow daily. The exact amounts were not measured but it was noted that all animals of this group gained weight of the order of 10–15 gm. over the 7–9 day period before sacrifice.

In Table 1 is shown data on the incorporation of radioactive acetate into liver fatty acids by slices from animals fed *ad lib.* It will be seen that in such animals growth hormone treatment caused a decreased incorpora-

[1] The growth hormone used in this study was obtained from the Connaught Laboratories, Toronto (Lot No. 200) through the offices of the National Research Council of Canada.

TABLE 1. THE EFFECT OF GROWTH HORMONE ON THE INCORPORATION OF 2-C^{14} ACETATE INTO FATTY ACIDS BY LIVER SLICES OF AD LIB FED INTACT AND ADRENALECTOMIZED RATS

Intact		Adrenalectomized	
% Incorporation per gm. liver		% Incorporation per gm. liver	
Control	+ Growth hormone	Control	+Growth hormone
6.9	3.0	3.0	1.01
5.9	2.7	2.3	0.98
4.5	2.6	1.2	1.47
8.1	5.2	1.0	0.98
4.8	2.3	2.5	1.54
3.3	1.2	2.8	0.84
		1.3	0.88
*5.6 ±0.70	*2.8 ±0.47	*2.0 ±0.46	*1.10 ±0.10
P = < .01		P = .05	

* = Mean ± Standard error.

tion of acetate into the liver fatty acids in both intact and adrenalectomized animals.

Similar results were obtained in force-fed adrenalectomized animals as is shown in Table 2. The extent of the inhibition of acetate by growth hormone was more marked than that observed with adrenalectomized rats fed *ad lib.* In considering the effect of growth hormone in the force-fed intact rats it will be seen that although growth hormone did inhibit fat formation in most experiments in others there was increased incorporation. In the group as a whole the difference in incorporation between control and treated animals was not significant.

In addition it will be noted that acetate incorporation by slices of both *ad lib.* and force-fed adrenalectomized animals was less than that of the appropriate controls. These findings are similar to results previously reported (Perry and Bowen, 1954). In all these experiments the fatty acid

TABLE 2. THE EFFECT OF GROWTH HORMONE ON THE INCORPORATION OF 2-C^{14} ACETATE INTO FATTY ACIDS BY LIVER SLICES OF FORCE FED INTACT AND ADRENALECTOMIZED RATS

Intact		Adrenalectomized	
% Incorporation per gm. liver		% Incorporation per gm. liver	
Control	+Growth hormone	Control	+Growth hormone
16.0	6.0	18.4	4.6
26.2	13.2	2.3	1.8
20.6	21.8	6.7	1.3
36.9	21.7	14.6	1.6
15.2	11.5	14.0	2.5
25.4	4.5	24.2	6.6
12.2	14.1	6.5	2.2
20.6	24.0		
*21.6 ±2.7	*14.6 ±2.6	*12.4 ±3.2	*2.9 ±0.74
P = > .05		P = < .01	

* = Mean ± Standard error.

content of the liver slices was between 2.7 and 3.2% with no differences noted between animals in the various groups.

DISCUSSION

Although the exact mechanism is not clear, there seems no doubt that growth hormone may inhibit the process of fatty acid synthesis by liver slices and further, such inhibition can occur in the absence of the adrenal cortex. Lipogenesis is depressed by adrenalectomy itself but a further depression occurs on administering growth hormone. Thus it would appear that this action of growth hormone, unlike its effect on R.Q. and muscle glycogen (Recant, 1952; Illingworth and Russell, 1951) is a primary one, it is not due to acting synergistically with the adrenal cortex nor does it appear that "permissive levels" of adrenal hormones are necessary for growth hormone to inhibit the process of lipogenesis. These findings are in agreement with a recent report by Greenbaum (1954) in which growth hormone was shown to inhibit the incorporation of pyruvate into liver fatty acids.

The finding of depressed acetate incorporation in the adrenalectomized animals is in agreement with previously reported data of ours (Perry and Bowen, 1954). A depressed incorporation of C^{14} glucose into liver fatty acids has also been noted in the adrenalectomized rat (Perry and Bowen, unpublished observations).

In some of the intact animals force-fed glucose, growth hormone appeared to stimulate lipogenesis. This is difficult to explain; it could conceivably be a manifestation of either the reported insulin-like action of growth hormone (Ottaway, 1951; Park *et al.*, 1952) or stimulation of pancreatic secretion of insulin by growth hormone (Millman and Russell, 1950).

The action of growth hormone in inhibiting lipogenesis might be considered a manifestation of the carbohydrate conserving action of this hormone. In its defective ability to synthesize fatty acids the adrenalectomized animal treated with growth hormone resembles the fasting intact and the diabetic animal. Carbohydrate is necessary for proper fat formation either directly or as an energy source. This carbohydrate prerequisite is lacking in the adrenalectomized animal due to defective gluconeogenesis and possibly inadequate intake. The carbohydrate conserved as a result of the action of growth hormone cannot play its part in lipogenesis. In the adrenalectomized animal this action of growth hormone is superimposed on an already existing lack of carbohydrate needed for proper lipogenesis, though in the adrenalectomized animal where the carbohydrate lack is partially corrected at least by forced feeding, growth hormone still markedly inhibits fat formation.

SUMMARY

The incorporation of acetate 2-C^{14} into fatty acids by liver slices from intact and adrenalectomized rats has been observed and the effect of growth hormone on this process studied.

Growth hormone was found to inhibit lipogenesis in both intact and adrenalectomized animals. In the latter it further depressed the low acetate incorporation already present in comparison with intact controls.

It was concluded that the presence of adrenal cortical hormones was not necessary for growth hormone to affect the process of fat formation.

Acknowledgment

This work was supported by a grant-in-aid from the National Research Council, Canada.

REFERENCES

BALMAIN, J. H., S. J. FOLLEY, R. F. GLASCOCK AND M. L. MCNAUGHT: *Biochem. J.* **56:** VI. 1954.

BRADY, R. O., F. W. LUKENS AND S. GURIN: *J. Biol. Chem.* **193:** 459. 1951.

FELTS, J. M., I. L. CHAIKOFF AND M. J. OSBORN: *J. Biol. Chem.* **191:** 683. 1951.

GREENBAUM, A. L.: International Symposium on Hypophyseal Growth Hormone, Detroit, Oct. 1954.

GREENBAUM, A. L. AND PATRICIA MCLEAN: *Biochem. J.* **54:** 413. 1953.

ILLINGWORTH, B. A. AND JANE RUSSELL: *Endocrinology* **48:** 423. 1951.

MILLMAN, A. E. AND JANE RUSSELL: *Endocrinology* **47:** 114. 1950.

OTTAWAY, J. H.: *Nature*, **167:** 1064. 1951.

PARK, C. R., D. H. BROWN, M. CORNBLATH, W. H. DAUGHADAY AND M. E. KRAHL: *J. Biol. Chem.* **197:** 151. 1952.

PERRY, W. F. AND HELEN F. BOWEN: *Amer. J. Physiol.* **180:** 21. 1955.

RECANT, L. *Fed. Proc.* **11:** 272. 1952.

WELT, J. D. AND A. E. WILHELMI: *Yale J. Biol. and Med.* **23:** 99. 1950.

ON THE FAILURE OF GLYCOGEN DEPOSITION IN THE LIVERS OF SCORBUTIC GUINEA PIGS[1,2]

E. M. NADEL, A. S. MULAY AND L. D. SASLAW[3,4]

Laboratory of Pathology, National Cancer Institute,[5] Bethesda, Maryland

INTRODUCTION

A DECREASED amount of glycogen occurs in the liver of the scorbutic guinea pig, and partly because of this it was suggested that decreased adrenocortical function exists in scurvy. The problem has been carefully reviewed by Morgan (1952). More recently, increased amounts of cortisol (Compound F), have been identified in the blood and urine of scorbutic guinea pigs (Burstein, Dorfman, and Nadel 1954), supporting the suggestion that adrenocortical hormone production was not diminished, but actually was enhanced in scurvy (Clayton and Prunty, 1951; Nadel and Schneider, 1951, 1952). Decreased glycogen in the livers of scorbutic guinea pigs in the presence of increased amounts of circulating cortisol appeared to be paradoxical to us, for it suggested that this glucocorticosteroid was not physiologically able to help deposit glycogen in the liver when the guinea pig was depleted of vitamin C (Nadel and Mulay, 1954). The problem was investigated further by comparing the abilities of vitamin C deficient guinea pigs, and of controls to deposit liver glycogen following intraperitoneal injection of known glycogen precursors.

EXPERIMENTAL

Procedure

Biological

All experiments were done with young adult male Hartley guinea pigs obtained from the colony at the Laboratory Aids Branch of the National Institutes of Health. The animals were housed singly, or in pairs, in cages with wire screen bottoms to prevent coprophagy.

Received for publication November 22, 1954.

[1] Submitted for publication.

[2] Presented in part at the 39th annual meeting of the American Society of Experimental Pathologists, April 14, 1954 in Atlantic City.

[3] Part of this work was offered as one of the requirements for the degree of Master of Science at the Columbian College of the George Washington University, Washington, D. C.

[4] Present address Laboratory of Chemical Pharmacology, National Cancer Institute.

[5] National Institutes of Health, Department of Health, Education, and Welfare. Public Health Service, Bethesda, Maryland.

Previous to the experiment, 119 guinea pigs were maintained on Purina Rabbit Chow Checkers (Complete Ration E),[6] fresh cabbage, and carrots daily *ad libitum*. All animals were fed water *ad libitum*. Thirty-eight guinea pigs, designated as *Normal Controls, Group I*, were starved 24 hours before the injection of the glycogen precursors. Forty-five guinea pigs, designated as *Starved Controls, Group II*, were starved for 4 days before the injections. During the starvation period, each guinea pig received 20 mg. of l-ascorbic acid daily given by mouth in 0.5 ml. of distilled water. (The four-day starvation period was selected because it caused a weight loss approximately equal to that experienced by the scorbutic animals.) Thirty-six guinea pigs, designated as *Scorbutics, Group III*, were transferred to a diet of Rockland Rabbit Ration[7] which they were fed *ad libitum* for the 22 to 28 days during which time late acute scurvy developed. The pigs were then fasted for 24 hours and injected as described below. Animals were weighed to the nearest gram at 4–5 day intervals during the course of the experiment and just prior to decapitation. The following solutions were prepared in distilled water on the morning of analysis: 5% d-glucose, 5% d-fructose, 5% dl-alanine and 5% sodium ascorbate. The sodium ascorbate solution was prepared by adjusing the pH of a freshly prepared l-ascorbic acid solution to pH 7.0 and then bringing the total volume to yield a concentration of 5%. Twelve to fifteen guinea pigs from the 3 groups were injected each day. All injections were administered intraperitoneally in 3 hourly doses so that each guinea pig received a total dose of approximately one mg. of indicated compound per gm. of body weight. Three hours after the last injection, each pig was stunned by a blow on the head and decapitated. A liver sample was taken for analysis, as below.

Chemical

The method of Good *et al.* (1933) slightly modified, was used to isolate and hydrolyze the liver glycogen. Analysis of glucose was conducted according to the method of Folin (1920) using a model B Beckmann spectrophotometer. Liver glycogen was expressed as milligrams of glucose per gram of liver. Statistical analysis of the data was done by the method of Mantel (1951).

RESULTS

The liver glycogen values for the 3 groups appear in Table 1 and Fig. 1, while the weight losses in the starved control group and scorbutic group appear in Table 2.

After the administration of *fructose or glucose*, the deposition of liver glycogen was lowest in the scorbutic group in comparison to that in the two control groups. Glycogen values in the starved controls were not significantly different from those in the normal controls. Within each group no difference could be detected between the effects of fructose and glucose on liver glycogen deposition.

After the administration of *alanine*, the deposition of liver glycogen was again lowest in the scorbutic group; within each group, deposition was significantly less after alanine than after fructose or glucose injection.

Untreated animals within each group showed significantly less liver gly-

[6] Distributed by Ralston Purina Co., St. Louis, Missouri.

[7] Distributed by Arcady Farms Milling Co., Chicago, Illinois. No difference was detected in glycogen deposition in normal controls with either diet supplemented with Vitamin C or with cabbage.

TABLE 1. LIVER GLYCOGEN DEPOSITION IN STARVED AND IN FASTED NORMAL AND FASTED SCORBUTIC (23 TO 28 DAYS) GUINEA PIGS 6 HOURS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF ASCORBIC ACID, ALANINE, FRUCTOSE OR GLUCOSE

Treatment	Series	*Group I* Normal controls (24-hr. fast)			*Group II* Starved controls (4-day fast)			*Group III* Scorbutics (24-hr. fast)		
		Liver glucose, mg./gm.	S.E.	No. of animals	Liver glucose, mg./gm.	S.E.	No. of animals	Liver glucose, mg./gm.	S.E.	No. of animals
None	a	1.63	0.9	6	4.22	0.5	10	2.54	1.3	8
Ascorbic acid	b	3.43	1.1	8	8.87	1.7	11	0.84	0.35	8
Alanine	c	9.58	1.5	8	11.77	1.4	8	1.45	0.5	6
Fructose	d	18.55	3.0	8	18.94	2.0	8	5.59	2.5	8
Glucose	e	18.57	3.1	8	17.67	1.5	8	4.26	1.4	6

Controls received ascorbic acid daily by mouth (20 mg. in 0.5 ml. distilled water. Injections were given intraperitoneally in 3 doses totalling 1 mg./gram. body weight.

There is a significant difference (p less than .01) between the following:
Group I, series a and b, versus c, d and e; Group II, series a and b versus c, d and e.
Group III, series a, b, and c versus d and e; Group II, series a versus b.
Group I, II and III, series a versus d and e; Series b, Group I and III versus II; Series c, Group I and II versus III.

cogen than animals treated with fructose or glucose. Untreated animals showed less deposition than alanine treated animals in normal and in the starved control groups, but no difference was present in the scorbutic group.

In *ascorbic acid* injected animals liver glycogen deposition was not significantly different from that in untreated animals within the normal control group or within the scorbutic group, but significantly less glycogen was deposited in these ascorbic acid treated animals than in animals receiving fructose, glucose or alanine. However, in the starved controls significantly more glycogen was deposited in animals injected with ascorbic acid than in the untreated animals. While this deposition was not significantly differ-

TABLE 2. AVERAGE FINAL BODY WEIGHTS AND AVERAGE PER CENT LOSS IN BODY WEIGHT OF THE STARVED CONTROLS AND FASTED SCORBUTIC (23 TO 28 DAYS) GUINEA PIGS

Treatment	Series	*Group II* Starved controls (4-day fast)				*Group III* Fasted scorbutics (24-hour fast)			
		Final body weight, gm.	S.E.	Body weight loss, %	S.E.	Final body weight, gm.	S.E.	Body weight loss, %	S.E.
None	a	216	7.8	26	1.0	198	10.0	26	2.1
Ascorbic acid	b	200	5.8	25	1.5	204	8.6	28	3.5
Alanine	c	218	3.6	23	1.5	209	13.9	28	6.0
Fructose	d	209	5.6	23	1.4	228	10.3	22	2.5
Glucose	e	220	4.6	21	1.4	210	13.8	23	3.3

The differences in the final body weights and the per cent body weight losses in the Starved Controls and the Fasted Scorbutics are not significant.

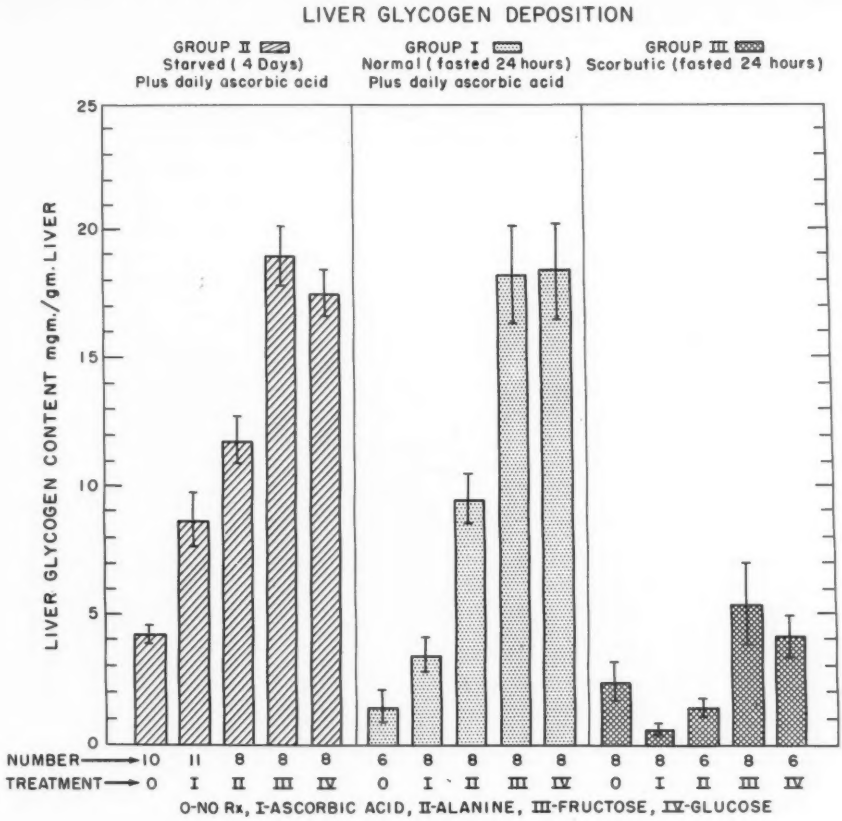

LIVER GLYCOGEN DEPOSITION
GROUP II
Starved (4 Days)
Plus daily ascorbic acid
GROUP I
Normal (fasted 24 hours)
Plus daily ascorbic acid
GROUP III
Scorbutic (fasted 24 hours)
LIVER GLYCOGEN CONTENT mgm./gm. LIVER
25
20
15
10
5
0
NUMBER → 10 11 8 8 8 6 8 8 8 8 8 8 6 8 6
TREATMENT → 0 I II III IV 0 I II III IV 0 I II III IV
O-NO Rx, I-ASCORBIC ACID, II-ALANINE, III-FRUCTOSE, IV-GLUCOSE

FIG. 1

ent from the deposition seen after alanine administration in starved controls, it was significantly less than the deposition following fructose or glucose injections.

DISCUSSION

A defect in the ability to form liver glycogen from precursors is clearly present in severely scorbutic guinea pigs. This defect is not related to the loss in body weight that occurs late in scurvy, for starved controls with similar weight losses deposited glycogen as capably as did normal controls.

Within each group no difference could be detected in deposition after fructose as compared to glucose. Thus while the scorbutic group showed less glycogen deposition than the controls, the defect in deposition was no greater after glucose than after fructose. This evidence suggests that probably no difference in the metabolism of their hexokinases (Chernick, Chaikoff and Abraham, 1951) occurs in scurvy. Murray (1948) has indicated

that the administration of insulin does not enhance liver glycogen deposition in scurvy.

Within each group less glycogen deposition was seen after alanine administration than after fructose or glucose and of the three groups, the scorbutic again was least able to deposit glycogen after alanine injection. Inasmuch as the metabolic pathway from alanine to glycogen is less direct than from glucose to glycogen, these results were to be anticipated.

The results indicate that ascorbic acid cannot be considered glycogenic for normal controls or for scorbutic animals. Not only was there no difference in glycogen deposition present in the untreated or in the ascorbic acid treated animals in these 2 groups, but significantly less liver glycogen was present after ascorbic acid injection than after the injection of alanine, fructose or glucose. However, more glycogen was deposited in the starved controls after the injection of ascorbic acid than after no treatment. This amount was close to the amount found in the livers of alanine tested animals but was significantly less than that seen in the fructose or in the glucose tested animals. We believe this observation to be not only statistically, but biologically significant as well. This belief is based on the observations of a significant increase terminally in cortisol excretion in starved animals despite the presence of daily ascorbic acid in the diet (Nadel and Schneider 1951, 1952 and unpublished; Burstein, Dorfman and Nadel, 1954). The presence of this endogenous increase in cortisol in starvation together with the large amounts of ascorbic acid injected (1 mg./gm. body weight) in this experiment may have permitted endogenous cortisol to exert its effect in converting body protein into glycogen. However, in the prolonged absence of ascorbic acid in the diet, even the greater amounts of cortisol that are produced endogenously in scurvy are relatively unable to convert either body protein or known glycogen precursors into glycogen within the 6 hour period of testing. We have also observed this defect persistent after the administration of cortisol or of cortisone (Nadel 1953, unpublished) as have Murray (1948) and McKee, Cobbey and Geiman (1949) after the administration of adrenal cortical extracts. However, the injection of large doses of ascorbic acid preceding the injection of cortisol or cortisone in scorbutic guinea pigs will cause an increased amount of liver glycogen to be deposited (Mulay, Saslaw and Nadel, 1954).

From these studies it is apparent that ascorbic acid is a necessary component in the production of liver glycogen in the guinea pig. Whether one of its functions is as an accessory factor or as a coenzyme influencing glucose metabolism or corticosteroid metabolism are questions that are raised by this study. However, the possible role of vitamin C in the metabolism of glucose and steroids cannot be properly defined by studies limited to mammals not requiring the vitamin as a constant part of their daily diet. In this cause the guinea pig, monkey and man are the only suitable mammalian species.

SUMMARY

The apparent paradox of decreased glycogen in the livers of scorbutic guinea pigs despite the presence of increased circulating cortisol (Compound F) was studied by comparing the abilities of Vitamin C deficient guinea pigs and controls to deposit liver glycogen following intraperitoneal administration of known glycogen precursors. Six hours after intraperitoneal administration of fructose, glucose or alanine, less glycogen was found in the livers of the scorbutic group in contrast to that found in the starved or normal groups. In each group less glycogen was found after alanine than after fructose or glucose. In each group no difference in glycogen deposition was detected after fructose as compared to glucose. The defect in the scorbutic group was not related to body weight loss, for the starved group, with a similar weight loss, deposited glycogen as capably as did the normal group. In contrast to that in untreated animals, more glycogen in each group was deposited after alanine, fructose and glucose. In the normal and in the scorbutic group the injection of ascorbic acid, in a fashion similar to the injection of the glycogen precursors, was not associated with an increase in glycogen in comparison to the untreated animals. However, following the administration of ascorbic acid to starved animals, an increase in glycogen over that of the untreated animals was observed. In addition to other possible alterations occurring in scurvy and involving the metabolism of carbohydrates, it appears that ascorbic acid may be a necessary accessory factor influencing the metabolism of glucose or glucocorticosteroids or both.

REFERENCES

BURSTEIN, S., R. I. DORFMAN AND E. M. NADEL: *Fed. Proc.* **13**: 188. 1954.
BURSTEIN, S., R. I. DORFMAN AND E. M. NADEL: *J. Biol. Chem.* 1954 (in press).
CHERNICK, S. S., I. L. CHAIKOFF AND S. ABRAHAM: *J. Biol. Chem.* **193**: 793. 1951.
CLAYTON, B. E. AND F. T. G. PRUNTY: *Brit. Med. Journ.* ii 129. 1951.
FOLIN, O.: *J. Biol. Chem.* **41**: 367. 1920.
GOOD, G. A., H. KRAMER AND M. J. SOMOGYI: *J. Biol. Chem.* **100**: 485. 1933.
MCKEE, R. W., T. S. COBBEY AND Q. M. GEIMAN: *Endocrinology* **45**: 21. 1949.
MANTEL, N.: *Amer. Statist.* **5**: 26. 1951.
MORGAN, A. F.: Vitamins and Hormones Vol. 9, 161, Academic Press, 1951.
MULAY, A. S., L. S. SASLAW AND E. M. NADEL: *J. Clin. Endocrinol. and Met.* **14**: 7, 1954.
MURRAY, H. C.: *Proc. Soc. Exp. Biol. Med.* **69**: 351. 1948.
NADEL, E. M. AND A. S. MULAY: *Fed. Proc.* **13**: 440. 1954.
NADEL, E. M. AND J. J. SCHNEIDER: *J. Clin. Endocrinol. and Med.* **11**: 791. 1951.
NADEL, E. M. AND J. J. SCHNEIDER: *Endocrinology* **51**: 5. 1952.
NADEL, E. M., S. BURSTEIN AND R. I. DORFMAN: *Clin. Proc.* **3**: 42. 1955.

EFFECT ON THE THYROID GLAND OF EXPERIMENTAL ALTERATION OF THE LEVEL OF CIRCULATING THYROXINE IN MICE WITH HETEROTOPIC PITUITARIES[1]

ROBERT O. SCOW AND MONTE A. GREER[2]

National Institute of Arthritis and Metabolic Diseases and National Cancer Institute,[3] Bethesda, Maryland

RECENTLY it was observed that intraocular pituitary implants maintained thyroidal I^{131} metabolism in hypophysectomized mice at a higher level than that in hypophysectomized controls. The pituitary implants, however, had no effect on the thyroid weight (Greer, Scow, and Grobstein, 1953). The purpose of the present study was to determine what effect altering the level of circulating thyroxine would have on the radioiodine metabolism and on the weight of the thyroid in implanted-hypophysectomized mice. The thyroxine level was lowered by feeding propylthiouracil in the diet and elevated by injecting thyroxine in an amount exceeding the daily requirement.

EXPERIMENTAL

The mice used in these experiments were F_1 derivatives of two closely inbred lines, BALB/c and C_3H. The hosts were females, two months old at the time of implantation, and the donors were of both sexes, 1–2 days old. A single pituitary was transplanted into the anterior chamber of one eye of each host. About one week later, implanted and control mice were hypophysectomized by the parapharyngeal approach. Total extirpation of the pituitary gland was achieved in all operated mice reported in this paper as ascertained at autopsy by visual inspection (9×magnification) of the sella turcica and adjacent tissues. Since there was no evidence of gonadotrophic hormone being secreted by the implant, the degree of uterine atrophy was used as an additional indication of completeness of hypophysectomy.

Two different diets were used in this study. One was a pelleted stock diet prepared by the Derwood Mill[4] and the other, diet #1, was a purified-type diet containing glucose (73%), casein (20%), fat (3%), salts (4%), and vitamins (basal diet #1 of Franklin et al. (1947) without succinylsulfathiazole). The pelleted diet was ground to a powder be-

Received November 29, 1954.

[1] A preliminary report of these results was presented at the Endocrine Society meetings in New York City, June, 1953 (Scow and Greer, 1953).

[2] We are indebted to Dr. Clifford Grobstein for making the intraocular transplants and to Mr. Howard Erwin for valuable technical assistance.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[4] Derwood diet composition: Skim milk powder, 22.75; ground whole wheat, 61.52; dried brewer's yeast, 4.00; cod liver oil, 2.00; NaCl, 1.40; ferric citrate, 0.13; and corn oil, 8.20 grams (Derwood Mill, Derwood, Md.).

fore adding the propylthiouracil. The level of propylthiouracil in the diet was 0.15 or 0.30%. Feeding of propylthiouracil was begun during the 3rd or 4th postoperative week. When the total I^{131} uptake was to be determined, propylthiouracil was removed from the diet 2–3 days prior to the test, whereas it was fed up to the test day for the thyroid/serum iodide ratio determinations. The thyroxine and KI injections, given intraperitoneally, were begun at the end of the fourth postoperative week. The daily dosage of thyroxine, dissolved in a slightly alkaline solution, was 5.0 μg. and that of KI, 4.3 μg.

The total thyroid I^{131} uptake was determined as follows: 4 or 4½ hours after injecting 1 μc. of carrier free I^{131} as NaI, each mouse was killed and the thyroid was removed and weighed. The thyroid/serum iodide (T/S) ratio was determined by comparing the radioiodide concentration in the thyroid with that in the serum. Each mouse received 2.5 mg. propylthiouracil subcutaneously 30 minutes before an injection of 1–2 μc. I^{131}. Two hours after the radioiodide injection, the animals were killed by exsanguination, a blood sample was collected from the heart, and the thyroid gland was removed. The radioactivity of thyroid gland and serum was determined by beta counting, as described earlier (Greer, Scow, and Grobstein, 1953).

RESULTS

Thyroid Weight: There was no significant difference in thyroid weight between implanted-hypophysectomized and hypophysectomized control mice (Figs. 1, 2, and 4). The thyroid weight in these animals averaged two-thirds of that in normal control mice.

Propylthiouracil feeding resulted in a slight increase in thyroid weight in implanted-hypophysectomized mice and a 3 to 4-fold increase in normal

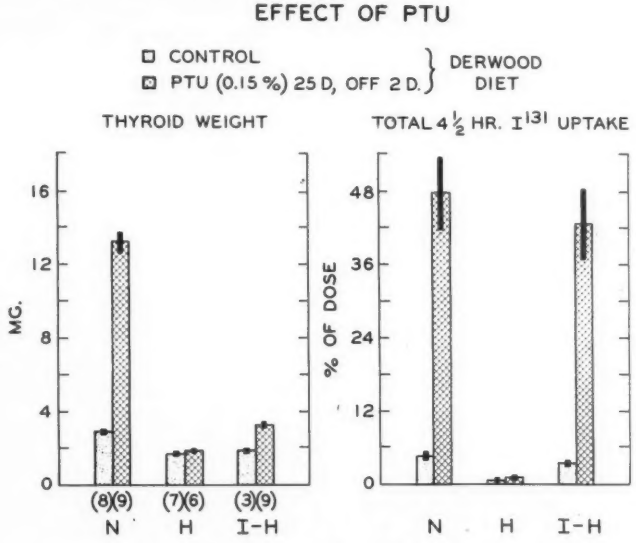


FIG. 1. Effect of propylthiouracil on total thyroid I^{131} uptake in mice fed Derwood diet. PTU, propylthiouracil; D, days; N, normal control; H, hypophysectomized control; I-H, implanted-hypophysectomized. Figures in parentheses indicate number of animals in each group. All groups were operated on, treated and tested together. The dark vertical bar represents the range of one standard error.

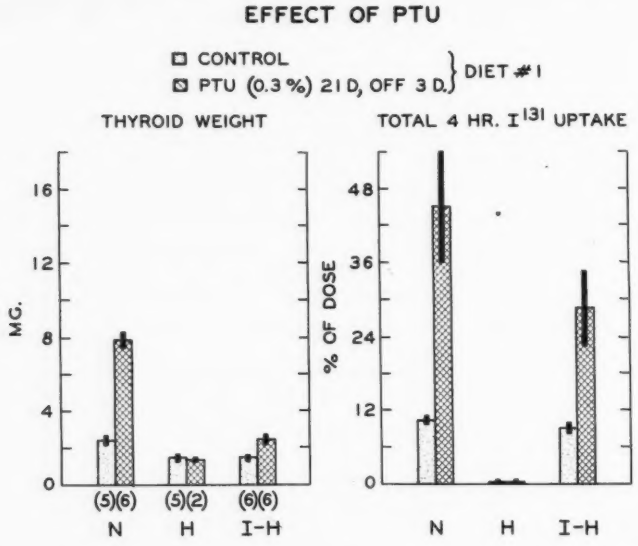


FIG. 2. Effect of propylthiouracil on total thyroid I^{131} uptake in mice fed diet #1. See Figure 1 for legend to abbreviations. All groups were operated on, treated and tested together.

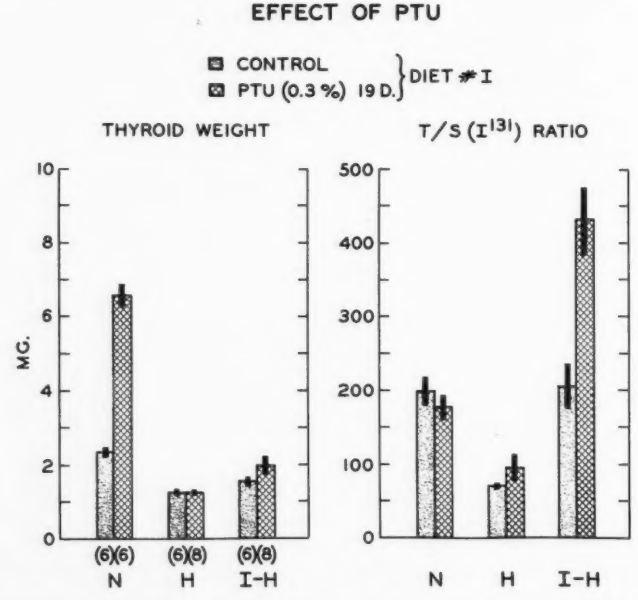


FIG. 3. Effect of thyroxine on total thyroid I^{131} uptake in mice fed Derwood diet. See Figure 1 for legend. The animals in the KI-treated group were operated on, injected and tested one week later than those in the thyroxine-treated group.

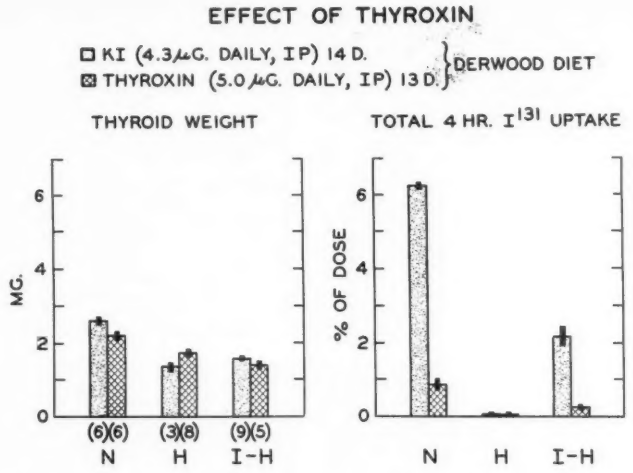


FIG. 4. Effect of propylthiouracil on the thyroid/serum iodide ratio in mice fed diet #1. See Figure 1 for legend. All groups were operated on, treated and tested together.

mice but had no effect in hypophysectomized controls (Figs. 1, 2, and 4).

Thyroxine injections for 13 days had no significant effect on thyroid weights in any of the three groups of animals (Fig. 3).

Thyroid I^{131} Uptake: The total thyroidal I^{131} uptake at 4 or $4\frac{1}{2}$ hours in implanted-hypophysectomized mice was nearly the same as that in normal control mice and was much greater than that in hypophysectomized controls (Figs. 1 and 2). Although the diets were not compared at the same time, it would appear that the uptake was greater in mice, except for the hypophysectomized controls, fed diet #1 than in those fed Derwood diet (Table 1).

Feeding propylthiouracil induced a marked increase in thyroid I^{131} uptake in implanted-hypophysectomized and in normal mice but was without effect in hypophysectomized controls. When the marked difference in thyroid weight was corrected for, the uptake per mg. of thyroid gland was 2 to 3.7 times as great in implanted as in normal control mice (Table 1).

Thyroxine injections reduced the thyroid I^{131} uptake in implanted hypophysectomized and in normal control mice but had no effect on uptake in hypophysectomized control mice (Fig. 3). The control animals for all groups of thyroxine-treated mice were injected daily with an amount of iodine as KI equal to that in the daily dose of thyroxine.

Thyroid/Serum Iodide Ratio: The thyroid/serum iodide ratio in implanted-hypophysectomized and in normal control mice was about 200, which was nearly three times as large as that in hypophysectomized control mice (Fig. 4).

Propylthiouracil feeding doubled the T/S ratio in implanted-hypophy-

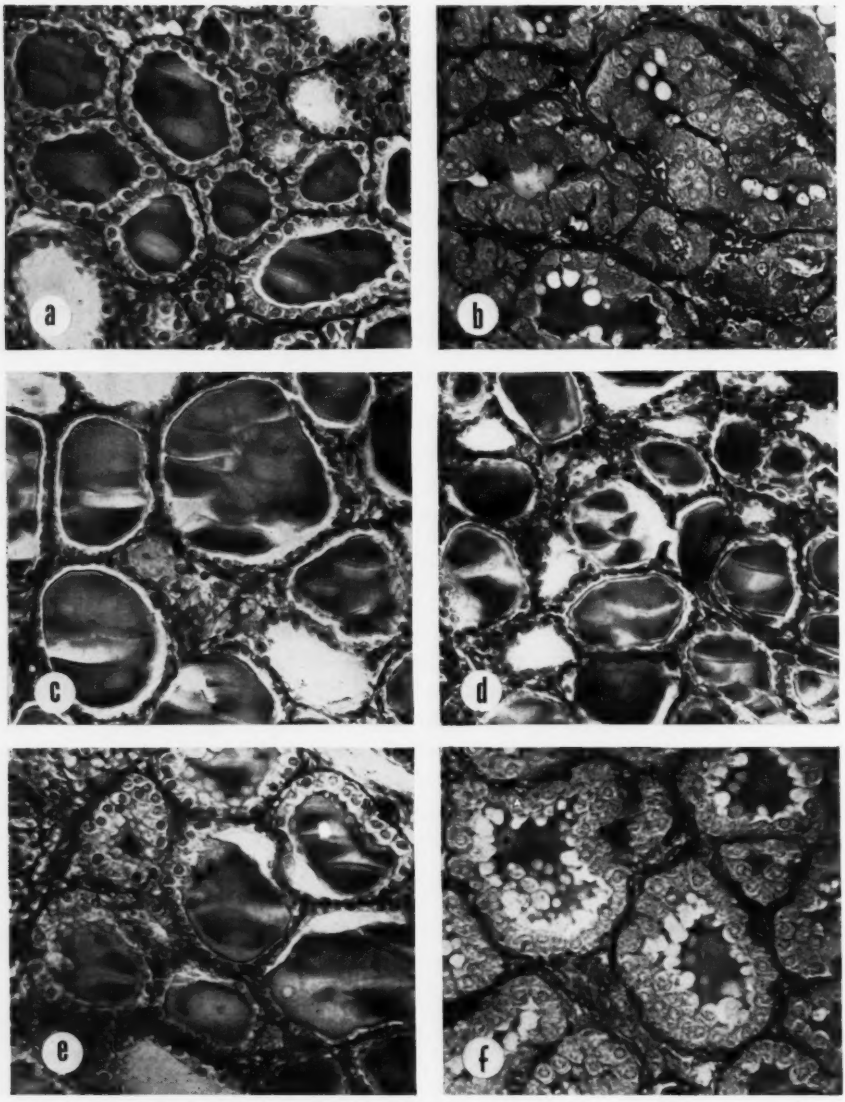


FIG. 5. Effect of feeding propylthiouracil on the cytology of the thyroid gland (0.15% PTU in Derwood diet for 3 weeks). (Modified Mallory triple stain; same magnification). a. Thyroid of untreated normal mouse. b. Thyroid of PTU-treated normal mouse. Note great increase in both nuclear and cell size, marked loss of colloid, and papillary infoldings of follicles. c. Thyroid of untreated hypophysectomized mouse. d. Thyroid of PTU-treated hypophysectomized mouse. No essential change is produced in the thyroid of the hypophysectomized mouse by feeding PTU. e. Thyroid of untreated implanted-hypophysectomized mouse. Cells intermediate between those of hypophysectomized and

TABLE 1. EFFECT OF FEEDING PROPYLTHIOURACIL ON THE TOTAL I^{131} UPTAKE[a] PER MG. OF THYROID GLAND

Diet	Derwood[b]		Diet #1[c]	
Time after I^{131} injection	4½ hr.		4 hr.	
Group	Untreated	PTU	Untreated	PTU
Normal	1.6 ± .3 (8)[d]	3.7 ± .5 (9)	4.4 ± .3 (5)	5.5 ± 1.1 (6)
Implanted-hypophysectomized	1.9 ± .2 (3)	13.7 ± 2.1 (9)	6.4 ± 2.1 (9)	11.1 ± 2.3 (6)
Hypophysectomized control	.6 ± .2 (7)	.3 ± .1 (6)	.2 ± .1 (5)	.2 ± .1 (2)

[a] % of I^{131} dose.
[b] Same animals as in Figure 1: PTU, .15%, fed 25 days and removed from diet two days before test.
[c] Same animals as in Figure 2: PTU, .30%, fed 21 days and removed from diet three days before test.
[d] Mean ± standard error; figures in parentheses indicate number of animals in each group.

sectomized mice but had no effect in normal and hypophysectomized control mice.

DISCUSSION

These results confirm our earlier report that intraocular pituitary implants maintained thyroidal radioiodine metabolism of hypophysectomized mice at a much higher level than that of hypophysectomized controls (Greer, Scow, and Grobstein, 1953). In the present experiment, the thyroxine level in the animal was raised by injecting thyroxine in an amount exceeding the daily requirement and was lowered by feeding propylthiouracil. The present data show that the thyroidal I^{131} metabolism of the implanted-hypophysectomized mice was depressed or augmented when the level of circulating thyroxine was raised or lowered, respectively. This change in functional capacity was accomplished without the corresponding marked change in thyroid weight which was produced in the intact control animals.

A definitive interpretation of the results is not possible with the data available. The intraocular pituitaries seem to be functioning at a submaximal rate in animals fed the basal diet. It would appear that their level of thyrotropin secretion can be raised or lowered by appropriately altering the level of circulating thyroxine, which in turn produces marked thyroidal metabolic changes, but with little change in the thyroid weight. Definite cellular changes in the thyroid gland are also produced in the implanted animals, as seen in Figure 5. It is not clear why the radioiodine metabolism

normal mice. f. Thyroid of PTU-treated implanted-hypophysectomized mouse. As seen in the treated normal, there is an increase in cell and nuclear size, some loss of colloid and some papillary indentation of follicles. With the exception of nuclear size increase, the response to PTU-treatment in the implanted-hypophysectomized was less than that in the normal mouse.

per unit thyroid weight in implanted-hypophysectomized mice should be stimulated to such a marked extent above that in similarly treated intact controls.

The earlier studies showed that when mice were fed a nongoitrogen diet, the T/S ratio and I^{131} uptake per mg. of thyroid in those with heterotopic pituitaries was two-thirds of that in normal controls (Greer, Scow, and Grobstein, 1953). In this series these parameters seemed equal in both groups. The reason for this change is not apparent. The only known difference between the experiments is that they were performed approximately one year apart.

It is rather surprising that the normal mice had no increase in their thyroid/serum iodide ratio with prolonged propylthiouracil feeding. In this respect there appears to be a considerable species difference between the mouse and the rat; the latter has a ten-fold increase in the thyroid/serum iodide ratio following chronic treatment with propylthiouracil. One possible explanation for this difference may be that the value begins at a much higher level in the mouse and cannot be increased appreciably in the normal animal.

SUMMARY

The thyroid I^{131} uptake and the thyroid/serum (T/S) iodide ratio were studied in hypophysectomized mice bearing intraocular pituitary implants. The I^{131} uptake and T/S ratio were higher in implanted-hypophysectomized mice than in hypophysectomized controls, even though the thyroid weights were the same. The total I^{131} uptake and T/S ratio in implanted-hypophysectomized mice were equal to those in normals whereas the thyroid weight was two-thirds of normal.

Feeding propylthiouracil resulted in a marked increase in the I^{131} uptake and the T/S ratio in implanted-hypophysectomized mice with a relatively small, but statistically significant, thyroid weight increase. The I^{131} uptake per mg. of thyroid gland and the T/S ratio were two to three times as large in implanted mice fed propylthiouracil as in similarly fed normal controls.

Thyroxine injections produced a depression of the I^{131} uptake by the thyroid in both implanted-hypophysectomized and normal animals but had no significant effect on the thyroid weight.

The thyroid glands of hypophysectomized control mice were unaffected by propylthiouracil-feeding and by thyroxine.

REFERENCES

FRANKLIN, A. L., E. L. R. STOKSTAD, M. BELT AND T. H. JUKES: *J. Biol. Chem.* **169**: 427. 1947.

GREER, M. A., R. O. SCOW AND C. GROBSTEIN: *Proc. Soc. Exper. Biol. and Med.* **82**: 28. 1953.

SCOW, R. O. AND M. A. GREER: *J. Clin. Endocrinol. and Metab.* **13**: 855. 1953.

INDEPENDENCE OF ADRENAL HYPERPLASIA AND GONADAL MASCULINIZATION IN THE EXPERIMENTAL ADRENOGENITAL SYNDROME OF FROGS[1]

CHIH-YE CHANG AND EMIL WITSCHI

Department of Zoology, State University of Iowa, Iowa City, Iowa

THE similarity of the estrogen-induced adrenal and gonadal effects in larval frogs with the characteristic picture of the so-called adrenogenital syndrome of human pathology was pointed out in an earlier publication (Witschi 1953). However, experiments then in progress and already referred to in the discussion of the same paper, furnish proof that in the case of the frogs the gonadal and the adrenal changes are independent parallel reactions to the estrogen administrations. The present paper gives the full account of the recent set of experiments.

METHODS

The experiments were started with a large lot of eggs of Rana pipiens and repeated with smaller batches of R. temporaria and R. sylvatica cherokiana. Since the results were identical, as far as the present work is concerned, no further distinction of the three species will be made in this report. Of about 1000 specimens at the beginning, one-third were hypophysectomized through removal of the hypophyseal placode at the late neurula stage. At the end of the metamorphic period 509 non-operated animals were preserved. Another 268 non-metamorphosing hypophysoprivic larvae were fixed about the same time as their intact parallel groups (average age 3–4 months). In addition 5 control frogs and 3 permanent larvae were kept to the age of 16 months. Attempts to maintain estrogen treated animals beyond metamorphosis were not successful. The survivors total 785; a mortality and accident loss approximating one-fifth is not excessive, considering the often severe experimental interferences during embryonic and larval development.

Hormone administrations[2] were started at the stage of closing gill sacs and continued until the time of fixation. Depending on the particular species, the treatments thus lasted from 2 to 5 months. Estradiol and Methyltestosterone were dissolved in the aquarium water at the concentrations listed in Table 1.

A visual expression for the condition, size, and activity of the adrenal glands was obtained by preserving a number of animals of each experimental group in Fleming's solution. It is believed that the intensity of the reductive potency of the cortical cells and therefore the density of metallic osmium deposits is a fair index of cortical activity. The sections were stained with safranin and light green and photographed through a red-infra-red filter.

Received December 18, 1954.

[1] Aided by grant G-184 from the National Science Foundation.

[2] The authors are indebted to Ciba Pharmaceutical Products, Inc., for the generous supply of Estradiol (Ovocylin) and Methyltestosterone (Metandren).

RESULTS

a. Control Series: The slight preponderance of females among the controls together with the occurrence of some intersexes (Table 1) proves that the frogs used belonged to semidifferentiated local varieties. In fact, the hermaphrodites are genetic males and indicate the drift from female to male by which normally the equilibrium of the sexes becomes established within a few months after metamorphosis. The 6 per cent deviation from the ideal 1:1 ratio is negligible in the present set of experiments. The data contained in Table 1, Series a, reveal that hypophysectomy has no influence on sex differentiation; nor does it change in any other way the course of development of the sex glands during the larval period. It is only months after metamorphosis that the gonadal development of unoperated controls outdistances that of the hypophysectomized permanent larvae.

TABLE 1. SEX DIFFERENTIATION IN NORMAL AND IN HYPOPHYSECTOMIZED TADPOLES TREATED WITH ESTRADIOL

Series	Hormone	Concentration, μg./liter	Intact group			Hypophysoprivic group		
			♀	⚥	♂	♀	⚥	♂
a	Control	—	110	7	81	62		42
b	Methyltestosterone	20			51			49
c	Estradiol	20	75			26		
d	Estradiol	100	71			28		
e	Estradiol	500	12	18	19	2	13	5
f	Estradiol	1000–5000			70			44

As previously reported (Witschi 1930), hypophysectomy of salamander larvae (Taricha torosa) results in distinct underdevelopment of the testes, a few months after metamorphosis of the controls, but retardation of the ovaries becomes noticeable only after about one year.[3] In the frogs the effects appear slightly earlier than in the salamanders. One year after metamorphosis the testes of leopard frogs have much enlarged and contain all stages of spermatogenesis, including mature sperms (Fig. 1), while fraternal hypophysectomized larvae still have testes of the size and structure of metamorphosing males. They contain only spermatogonia (Fig. 2). The ovaries of 18 months old hypophysectomized larvae (Fig. 4) are nearly twice the size normally attained at metamorphosis; but they are very much smaller than those of unoperated sister frogs (Fig. 3). These conditions agree closely with those described by Fugo (1940) for hypophysecto-

[3] In parabiotic twins of Taricha also a separate effect on inductor capacity was observed which, however, becomes not manifest in experiments of the type here reported.

mized chicks. Of human hypophysoprivi it is reported that they are more often of female than male sex. But this imbalance is more likely a result of selection than sex reversal. Remarkable is the absence of oviducts in the hypophysoprivic larvae, while they are fully formed, convoluted and partly distended in the intact controls (Figs. 3 and 4). The *adrenals* of hypophy-

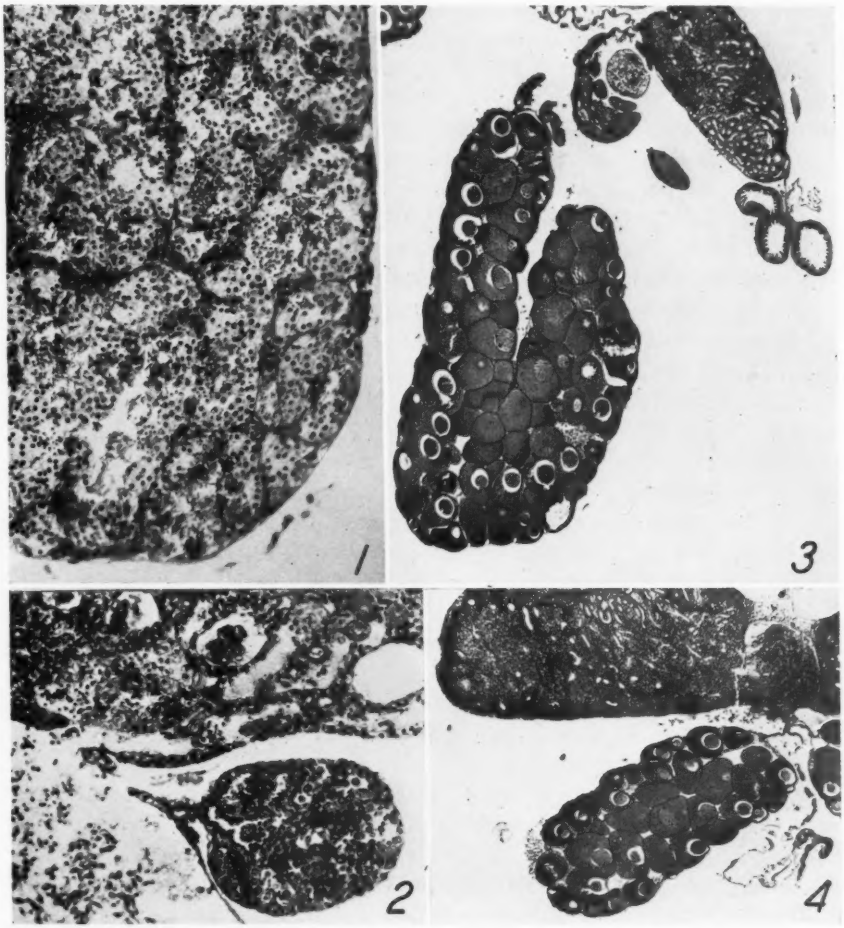


FIG. 1. Rana pipiens, 485 days old male frog. Part of a cross section through a testis, presenting all stages of spermatogenesis; ×100.

FIG. 2. Fraternal perennial larva (hypophysoprivus) of the former, 472 days old. Cross section through a testis, showing only primary spermatogonia; ×100.

FIG. 3. R. Pipiens 485 days old female frog. Cross section through ovary, mesonephros, and oviduct (convoluted, thrice cut); ×30.

FIG. 4. Sister larva (hypophysoprivus) of the preceding frog, 472 days old. Note smaller ovary, absence of the oviduct; ×30.

soprivic animals are distinctly underdeveloped, their volume being reduced to about one-fourth that of controls (compare Figs. 5 and 6). Closer examination proves that both the cortical and the medullary components are retarded. Reconstructions from serial sections indicate that also the entire chain of sympathetic ganglia is affected, lagging in growth behind that of controls (Chang, unpubl.).

In conclusion, total elimination of the buccal hypophysis by removal of the primordial placode does not interfere with the embryonic differentiation of either the adrenals or the sex glands; but it delays their later growth and functional development. The effects become first noticeable in the adrenals, later in the testes and last in the ovaries.

b. Methyltestosterone Series: The well-known masculinizing effect of androgens on gonad differentiation in frogs appears unchanged in the hypophysectomized group (Table 1, Series b). In earlier experiments (Witschi 1951) it had been found that over the wide range from 0.01 μg. per liter to 5 mg. per liter methyltestosterone causes genetic females to differentiate testes instead of ovaries. In the present investigation only one dosage level was tested (20 μg. per liter); but the clear-cut result permits the general conclusion that masculinization is a direct gonadal effect not mediated by either hypophysis or adrenal. This outcome is not unexpected, since Mintz and Martindale Foote (1947) had already observed sex reversal in female bullfrog larvae by testosterone propionate injections after late hypophysectomy. Analogous experiments with Pleurodeles (Mintz and Gallien 1954) confirm the conclusion that sex reversal is not mediated by the hypophysis.

The *adrenals* of this series are not sensibly different from those of controls.

c–f. Estradiol Series: The data assembled in Table 1, Series c–f, show clearly that hypophysectomy is without effect on sex differentiation. Concentrations of estradiol from 20 to 100 μg. per liter are feminizing the sexual differentiation of the genetic males, as indicated by the exclusively female composition of the experimental groups. On the other hand all normal as well as hypophysectomized larvae reared in high concentrations of the same hormone turn out to be males. The testes show many irregularities and usually are rather small. Occasionally intersexual traits are recognizable though the testicular character is always dominant. Evidently the genetic females have acquired testicular gonads, notwithstanding their inherited constitution. For the species pipiens, the turning point between feminizing and masculinizing action lies at about the concentration of 500 μg. per liter. At this level intersexes as well as males and females are found in both, the normal and the hypophysectomized groups.

The *adrenals* of all injected hypophysectomized groups are as underdeveloped as those of hypophysectomized controls. Segal (1953) has given a detailed description of the development of the adrenals of normal and

estradiol injected larvae. The condition attained in controls at the time of metamorphosis is well represented by Figure 5. From the place of origin in the mesial mesonephric flange, wedged between descending aorta and caval vein, each adrenal descended along the lateral wall of the caval vein and

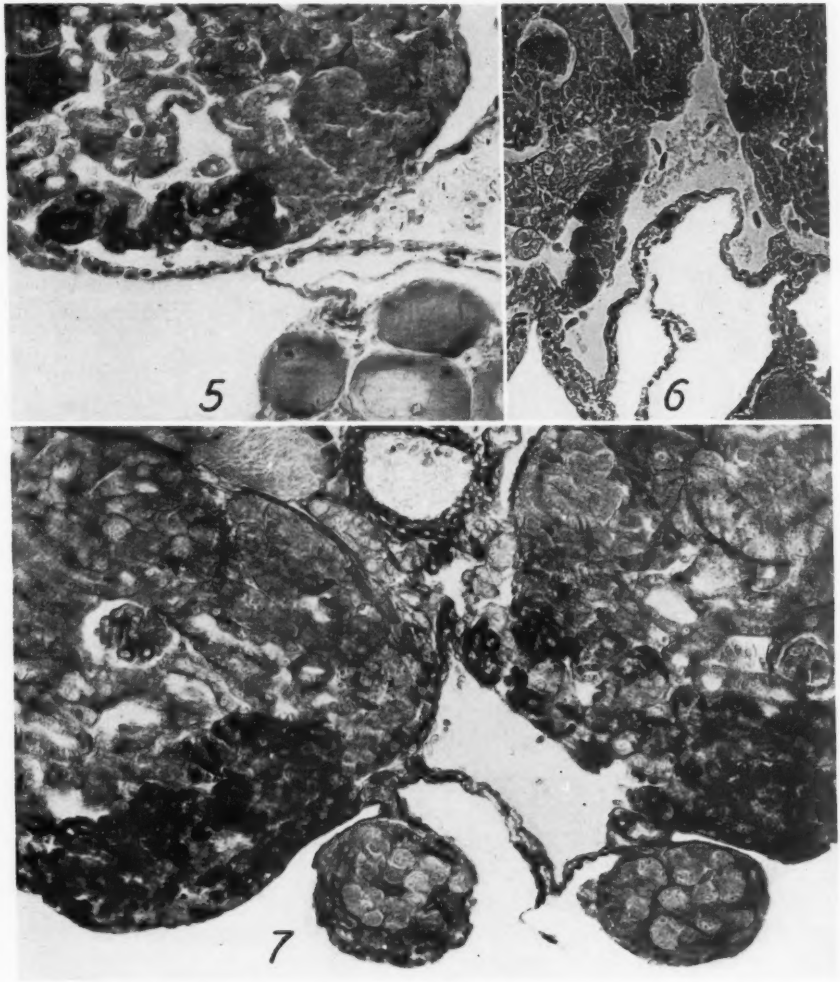


FIG. 5. Rana pipiens, metamorphosing control female, 4 months old. Representative cross section through the right adrenal. Cortical cells crowded with osmium deposits; medullary cells light; ×200.

FIG. 6. Non-metamorphosing, hypophysoprivic sister larva, 4 months old. Cross section through much retarded adrenals; ×200.

FIG. 7. R. pipiens metamorphosing frog, 4 months old, from group with high dosage estrogen treatment. Cross section through enlarged adrenals. Note continuing supply with medullary cells from the ganglionic layer around the dorsal aorta; ×200.

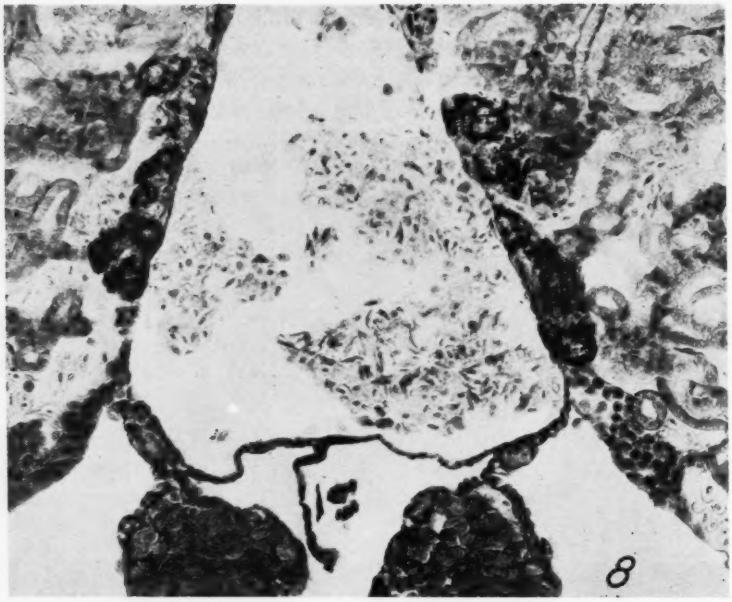


FIG. 8. Non-metamorphosing, hypophysoprivic larva of same species, 4 months old, preserved after injection of totally 1 mg. Armour ACTH over a period of 2 weeks; ×200.

is now located at the ventral surface of the mesonephric body. Osmicated sections give a fair picture of the size and activity of the adrenal gland. While only the cortical cells are impregnated, the outline of the entire gland is well recognizable, since the medullary cells are characteristically mingled with the cortical cords. In comparison, the adrenal of hypophysectomized control and estrogen-treated animals is much smaller; its location bordering the lateral wall of the caval vein, is nearer the place of em-

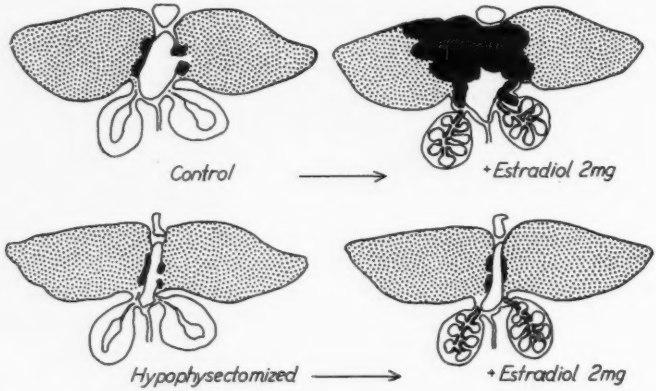


FIG. 9. Graphic summary of the major experiments.

bryonic origin and indicates therefore a slowing of the migration process (Fig. 6). Osmication produces only a fine, dust-like precipitation in the cortical cells.

Thus, estrogen-induced hyperplasia is restricted to animals still in possession of their hypophyses (Fig. 7), or, in other words, adrenal hyperplasia is an indirect reaction, mediated by the hypophysis. This conclusion is sustained by the fact that adrenals of hypophysectomized larvae are easily restored to normal size and activity through the administration of ACTH[4] (Fig. 8). However, an injection period of two weeks (total of 1 mg. Armour ACTH in 7 injections) is not sufficient to promote sensibly the ventrolateral migration of the adrenals (compare Fig. 8 with Figs. 5 and 6).

Figure 9 represents graphically the results of the experimental series.

DISCUSSION

It is evident that in the frog sex reversal and adrenal hyperplasia are independent reactions to estradiol administrations. However, this clear-cut result serves only as a prolegomenon to the more fundamental problems of the experiment.

Sex Differentiation: As pointed out elsewhere (Witschi 1954) it would seem fully as logical to prognose a masculinizing effect of estrogens on sex differentiation, as a feminizing one. In the dosage series reported in Table 1 estradiol obviously acts at the lower dosage ranges by suppressing the antagonistic medullary inductor; at the highest concentrations, a restraining effect on the homologous, i.e., the cortical inductor seems to gain prevalence. This latter effect is neither singular nor is it adequately characterized by the adjective "paradoxical." Administration at high dosages of almost any hormone sooner or later develops depressing effects on the homologous endocrine organ. Recent work proves that not the general principles, but their special applications change from one experiment to another (Mintz and Gallien 1954, Bruner and Witschi 1954). For the more penetrating analysis of the here presented series of experiments it seems important that not all estrogens act like estradiol. Equilenin, as estradiol, begins to show a feminizing influence at the level of about 10 μg. per liter, but even at 1000 μg. per liter it is still completely feminizing the genetic males (of R. sylvatica and R. temporaria). It should be added that equilenin produces adrenal hyperplasia, like estradiol. With the synthetic estrogens, stilbestrol, benzestrol, and fenocyclin, neither adrenal hyperplasia nor feminization or masculinization have so far been obtained, in spite of extensive experimentation. This is the more surprising as at least the first of these mimetic estrogens is known to have a partially feminizing influence on gonadal differentiation in birds (Boss and Witschi 1947) and in Xenopus (Segal, unpubl.). If selectivity of the gonad primordia alone should deter-

[4] Armour preparation #252-254-S3, obtained by courtesy of Dr. Irby Bunding, The Armour Laboratories.

mine the type of response, a more penetrating analysis would be a very difficult task. However, there remains at least a possibility that estradiol, if administered in high doses, might become a source of production of a hormone with androgenic properties. The synthesis would have to depend on the specific constitution of the estradiol-estrone-estriol group rather than on general estrogenicity, since equilenin does not bring forth this particular reaction. Almost certainly it would have to occur in an organ other than the adrenal. At any rate the hyperplastic adrenal does not contribute any extra androgen. This can be concluded from the fact that at the critical 500 μg. level (Table 1, Series e) the intact group is not shifted more definitely to the male side than the hypophysectomized group. The conditions under which adrenals may become androgenic are obviously not realized in the present experimental setup.

Adrenal Hyperplasia: A comparison of the results of our amphibian experiment with the complex situation as presented by the numerous reports on mammalian work meets with many difficulties. There is, of course, a close resemblance with the well known normal hyperplasia of the adrenals in human fetuses. The assumption that it is a consequence of the influx of placental estrogenic hormones seems well founded; and the fact that anencephali do not show the excessive growth constitutes good evidence that the hyperplasia in normal fetuses is controlled by way of their hypophysis glands. The human and the amphibian case have also in common that the effect is mostly a morphogenic one, developing at embryonic stages, and giving no evidence of androgenicity. A considerable hypertrophy of the adrenal cortex of adult rats after prolonged estrogen treatments was reported from many sides, and it was pointed out (Selye and Albert 1942) that the response shows the character of an alarm reaction. Since here again the effect does not develop in hypophysectomized animals, it seems assured that the estrogen stimulates directly the release and production of ACTH by the hypophysis. Bourne and Zuckerman (1941) established that the adrenal weight is greater in the estrous than the non-estrous rats. A similar peak is reached by the adrenal-weight-curve in pigeons (Riddle and Honeywell 1923) at the ovulation period. The accompanying rise in blood sugar suggests a concomitant out-pouring of glucocorticoids.

On the basis of these few pieces of evidence chosen from other vertebrate classes, we may generalize that the natural estrogenic hormones influence hypophyseal functions not only by controlling the rate of gonadotrophin secretion, as now usually taught, but that in addition they affect also the emission of ACTH. FSH and LH regulation by response of the hypophysis to blood-estrogen-levels is of well recognized importance for the maintenance of reproductive cycles. The biologic significance of the similar ACTH coordination is less obvious. From the fact that evidence of the same kind of interrelationship is found in mammals, birds, and amphibians, one must infer that it plays its part in some process of equally

widespread occurrence—obviously one related to female sex functions. At the present it seems most likely that the estrogen-ACTH-corticoid chain reaction, culminating in a metabolic crisis which resembles an alarm reaction, may sustain the ovulation process. This tentative interpretation of the adrenal response to estrogens is, of course, in need of confirmation by experiments bearing directly on the ovulation process.

SUMMARY

1) The effect of steroid hormones on adrenal and gonadal development was studied in parallel groups of intact and hypophysoprivic frog larvae.

2) In comparison with controls all hypophysoprivi have much reduced adrenals. Methyltestosterone administrations do not obviously change size or structure of the adrenals. Estradiol and Equilenin produce an extensive adrenal hyperplasia in intact larvae.

3) Ablation of the hypophysis does not affect sexual differentiation during the standard larval period (3 to 4 months).

4) Estradiol and methyltestosterone modify sex differentiation in hypophysoprivic exactly as in intact animals.

5) It is concluded that under the conditions of the present experiment the hyperplastic adrenal has no androgenic functions. Adrenal hyperplasia and sex reversal are independent responses to the estradiol administrations.

REFERENCES

Boss, W. R. and E. Witschi: *J. Exp. Zool.* **105**: 61. 1947.
Bourne, G. and S. Zuckerman: *J. Endocrinology* **2**: 283. 1941.
Bruner, J. A. and E. Witschi: *Anat. Rec.* **120**: 99. 1954.
Fugo, N. W.: *J. Exp. Zool.* **85**: 271. 1940.
Mintz, B. and L. Gallien: *Anat. Rec.* **119**: 493. 1954.
Mintz, B. and F. Martindale Foote: *Anat. Rec.* **99**: 19. 1947.
Riddle, O. and H. E. Honeywell: *Am. J. Physiol.* **66**: 340. 1923.
Segal, S. J.: *Anat. Rec.* **115**: 205. 1953.
Selye, H. and S. Albert: *Proc. Soc. Exp. Biol. and Med.* **50**: 159. 1942.
Witschi, E.: *Proc. Soc. Exp. Biol. and Med.* **27**: 763. 1930.
Witschi, E.: *Recent Progr. Horm. Res.* **6**: 1. 1951.
Witschi, E.: *Jour. Clin. Endocr. and Metab.* **13**: 316. 1953.
Witschi, E.: *Jour. Clin. Endocr. and Metab.* **15**: May. 1955.

NOTES AND COMMENTS

THE EFFECT OF PROPYLTHIOURACIL ON THE GROWTH AND THYROID: SERUM IODIDE RATIO OF MOUSE THYROID AUTOTRANSPLANTS

Morris and Green (1951) produced, in the mouse, a series of lines of transplantable tumors by heterologous transplantation of thyroid tissue chronically stimulated with thiouracil. It is pertinent to this oncological experimental approach to ascertain to what extent transplantation, *per se*, alters tissue function. Wollman and Scow (in a reference to unpublished data cited in Wollman, *et al.*, 1953) state that in the rat the autotransplanted lobe at a subcutaneous site shows a 30% decrease in function as compared with the intact lobe when the measure of function was I^{131} uptake. However, they do not give the conditions under which these observations were made. Rupp (1952), using other criteria of function, found no evidence of decreased thyroid function in the rat. He used an intrasplenic site for the autotransplant and the high vascularity of this site may have prevented tissue changes.

METHODS AND RESULTS

Twenty C3H/HeN mice, obtained from NCI stock, were fed a low iodine diet containing 0.025% PTU for 1 month. At the end of this period, the hyperplastic right lobe was transplanted to the right axilla. The animals were maintained on the goitrogen containing diet until 24 hours prior to sacrifice, when they were placed on the low iodine diet. The mice were

TABLE 1. THE EFFECT OF AUTOTRANSPLANTATION OF A THYROID LOBE TO A SUBCUTANEOUS SITE ON THE GROWTH RESPONSE TO PTU AND THE TRAPPING OF I^{131}

Months after autotransplant	No. of animals	Weight of intact lobe mg.	T/S of intact lobe	No. of functional transplanted lobes	Weight of transplanted lobe mg.	T/S of transplanted lobe
1	5	12.82± .80	179.1 ±11.4	4	1.91±0.45*	170.5±18.0*
2	6	14.46±1.58	197.71±10.7	6	2.99±0.78	177.0±22.5
3	9	17.16±2.34	195.1 ± 5.8	9	8.86±1.48	211.8± 9.3

* Mean and standard error of the mean (S.E.).

$$\text{S.E.} = \frac{\text{(highest—lowest) value}}{\text{number of animals}}\text{; Mantel (1951)}$$

divided into 3 groups, and the animals of each group were sacrificed at intervals of one, two and three months, respectively. After sacrifice the thyroid lobes were dissected free of other tissues under a dissecting microscope at 9× magnification. The activity of the thyroid iodide trap (T/S) was determined by the method of Halmi *et al.* (1953). The results as presented in Table 1 indicate that transplantation of one lobe of the thyroid to the axilla did not affect its ability to trap iodide. The tissue also showed the expected growth response to a goitrogen despite its presence in a strange site.

DISCUSSION

The functional status of the mouse thyroid gland, as measured by two criteria, iodide trap activity and growth response to goitrogen ingestion, is not influenced by the subcutaneous transplant site. The average transplant lobe weight was 50% greater 2 months after transplantation than the average weight obtained 1 month after transplantation. The weight increased by a factor of 3 between the second and third months of transplantation (Table 1), but the average weight of the intact lobes increased by less than

Received September 16, 1954.

50% between the first and third months. We think that this greater effect on the weight of the transplanted lobes was related to an increasing improvement in blood supply following transplantation. It, therefore, seems probable that at least 2 conditions must be met to insure optimal function: an adequate blood supply and sufficient time for the establishment of this blood supply.

SUMMARY

Autotransplantation of mouse thyroid lobes to a subcutaneous site did not change the activity of the iodide trap or modify the growth response to PTU over a 3 month period following the operation.

H. J. LIPNER,[1] BILLIE P. WAGNER AND HAROLD P. MORRIS[2]

National Cancer Institute
Public Health Service
Department of Health, Education, and Welfare
Bethesda, Maryland

REFERENCES

HALMI, N. S., B. N. SPIRTOS, E. M. BOGDANOVE AND H. J. LIPNER: *Endocrinology* **52**: 19. 1953.

MANTEL, N.: *Amer. Statistician* **5**: 26. 1951.

MORRIS, H. P. AND C. D. GREEN: *Science* **114**: 44. 1951.

RUPP, J. J.: *Endocrinology* **51**: 306. 1952.

WOLLMAN, S. H., R. O. SCOW, B. WAGNER AND H. P. MORRIS: *J. Nat. Cancer Inst.* **13**: 785. 1953.

[1] Public Health Service Fellow at the National Cancer Institute. Present address: Chicago Medical School, Chicago, Ill.

[2] The technical assistance of Mr. A. S. Gardner is gratefully acknowledged.

INHIBITION OF THE RELEASE OF THYROIDAL RADIOIODINE IN THE RAT BY CORTISONE

Cortisone has been shown to depress at least some aspects of thyroid activity. Perry (1951) and Albert, Tenney and Ford (1952) showed that the uptake of I^{131} by the thyroid gland of the rat was reduced by cortisone, but failed to obtain any effect on the rate of release of I^{131} from the thyroid. In view of the findings of Myant (1953) and of Brown-Grant, von Euler, Harris and Reichlin (1953) that cortisone administration reduced the rate of release of thyroidal radioiodine in the rabbit, it seemed worthwhile to reinvestigate the effect of cortisone in the rat.

MATERIALS AND METHODS

Eight adult female rats, body weight 202 gm. (± 5, standard error of mean), of an inbred laboratory colony, derived from the Lister strain, were used. The animals were fed a standard pellet diet (Medical Research Council Diet No. 41, containing approximately 230 μg. Iodine/Kg.) and tap water *ad lib.*, and were kept in a temperature-controlled animal room (27° C.) under constant lighting conditions. The animals were injected with 6 μc. of carrier-free radioiodine subcutaneously. Forty-eight hours later, twice daily *in vivo* counts of thyroid radioactivity were begun. Counts were made on unanaesthetised animals according to the technique of Fish, Carlin and Hickey (1952). The average uptake at 48 hours after injection was

Received October 8, 1954.

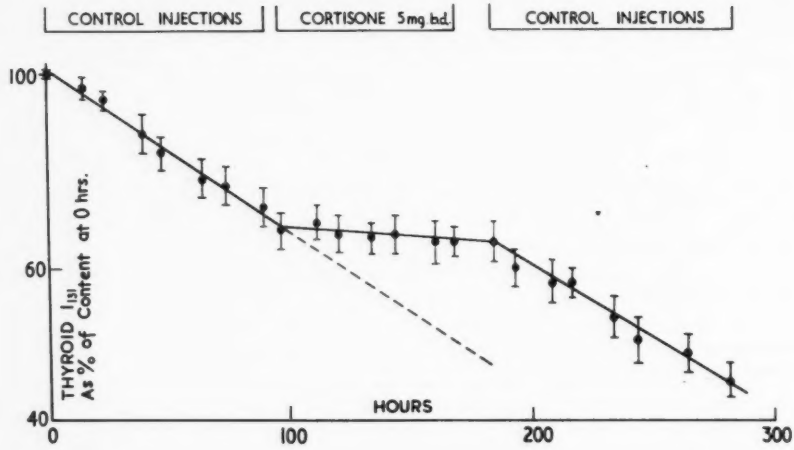


FIG. 1. To show the effect of cortisone (5 mg. twice daily subcutaneously) on the rate of release of thyroidal radioiodine in the rat. Each point represents the average of values for eight rats; vertical bars indicate standard error of mean. Control injections were given at the time of each count from 0–90 hours, cortisone (5 mg. b.d.) from 97–168 hours, and control injections again from 184–282 hours.

26.1 (±2.1, standard error of mean) % of the administered dose. The statistical error of counting at the rates observed was between ±2.0 and ±4.0%. The initial count at 48 hours after injection was taken as 100% and subsequent counts, after correction for isotope decay, were expressed as a percentage of the initial count. The average for all eight rats was calculated and plotted semi-logarithmically against time.

At the time of each count from 0 to 90 hours each animal was injected subcutaneously with 0.25 cc. of the aqueous vehicle used in the preparation of the cortisone suspension. At the time of the 97, 112, 121, 135, 144, 160 and 168 hour counts, each animal received 5 mg. of cortisone[1] (in a volume of 0.2 cc.) subcutaneously. The animals subsequently reverted to control injections for the third phase of the experiment.

RESULTS

As will be seen from Figure one, the injection of cortisone, 10 mg./day, produced a prompt, marked and reversible reduction in the rate of release of thyroidal radioiodine.

DISCUSSION AND SUMMARY

Cortisone, 5 mg. twice daily, reduces the rate of release of thyroidal radioiodine in the rat. The response is very similar to that seen in the rabbit (Brown-Grant, Harris and Reichlin, 1954). Previous workers had failed to demonstrate this effect in the rat. Perry (1951) and Albert *et al.* (1952) suggested that because the uptake of I^{131} by the rat thyroid was reduced, without the rate of release being affected, cortisone could not be acting via a depression of pituitary thyrotropic hormone secretion. In view of the findings reported here and the known effects of cortisone in the rabbit, this mechanism cannot be excluded on these grounds as a possible explanation of the influence of large doses of cortisone on thyroid function.

---

[1] Cortisone acetate (Roussel Laboratories, London) was supplied by the British Medical Research Council.

Acknowledgments

My thanks are due to the British Medical Research Council for the grant of a Research Studentship, during the tenure of which this work was performed.

K. BROWN-GRANT

Department of Neuroendocrinology
Institute of Psychiatry
Maudsley Hospital
London, England

REFERENCES

ALBERT, A., A. TENNEY AND E. FORD: *Endocrinology*, **50**: 324. 1952.
BROWN-GRANT, K., C. VON EULER, G. W. HARRIS AND S. REICHLIN: *J. Endocrin.* **9**: xliii. 1953.
BROWN-GRANT, K., G. W. HARRIS AND S. REICHLIN: *J. Physiol.*, **126**: 41. 1954.
FISH, W. A., M. CARLIN AND F. C. HICKEY: *Endocrinology*, **51**: 282. 1952.
MYANT, N. B.: *J. Physiol.*, **120**: 288. 1953.
PERRY, W. F.: *Endocrinology*, **49**: 284. 1951.

LIPOGENESIS AND CO_2 FORMATION FROM ACETATE IN THE LIVER OF THE HYPOPHYSECTOMIZED RAT[1]

In the present report we have compared lipogenesis in the livers of normal and of hypophysectomized rats. It is shown that the conversion of acetate to fatty acids is depressed in the liver of the hypophysectomized rat. A depression was observed as early as 14 hours and as late as 175 days after the complete removal of the gland.

EXPERIMENTAL

Rats of the Long-Evans and Sprague-Dawley strains were used. They were hypophysectomized by the ventral parapharyngeal route while under ether anesthesia. Completeness of hypophysectomy was confirmed at necropsy in all rats recorded here. The rats referred to as sham-operated were subjected to all operative procedures including manipulation of the pituitary gland but without its removal. All rats, normal hypophysectomized, and sham-operated, were fed throughout, *ad libitum*, a diet with the following composition: ground whole wheat, 67.5%; casein (technical), 15.0%; skimmed milk powder, 7.5%; sodium chloride, 0.75%; calcium carbonate, 1.5%; melted fat, 6.8%; fish oil (vitamins A and D concentrate), 1.0%; and a few drops of a KI solution.

The rats were sacrificed by cervical fracture, and the livers were quickly excised, weighed, and placed in ice-cold Krebs-Henseleit buffer (1932). The slices were prepared freehand, with a razor blade, to a thickness of approximately 0.5 mm.; 500-mg. portions were transferred to the 50-ml. incubation flask, and incubated in a reciprocating, shaking water bath at 37.5° C. for 3 hours. The details of the incubation procedure, a description of the incubation flask, and the methods used for determination of fatty acid-C^{14} and $C^{14}O_2$ have been recorded elsewhere (Felts, Chaikoff and Osborn, 1951; Baruch and Chaikoff, 1954; Henriques, 1946).

Received for publication December 4, 1954.
[1] Aided by a grant from the National Vitamin Foundation.

RESULTS

Table 1 shows the fatty acid-C^{14} and $C^{14}O_2$ recoveries found in experiments with liver slices prepared from normal, hypophysectomized, and sham-operated rats. The hypophysectomized rats were sacrificed from 14 hours to 175 days after excision of the pituitary gland. None of the rats lost more than a few grams following hypophysectomy, and most of them maintained their preoperative weights. All rats had access to the diet until the time they were sacrificed, and at necropsy their stomachs and small intestines were found to contain food.

The fatty acid-C^{14} recoveries observed in 6 of the 7 hypophysectomized rats (Table 1) ranged from 0.1 to 0.5% of the added C^{14}. In the case of rat no. 22, which was sacrificed 14 hours after hypophysectomy, the fatty acid-C^{14} recovery was 1.4%. The re-

TABLE 1. CONVERSION OF C^{14} OF ACETATE-1-C^{14} TO CO_2 AND FATTY ACIDS BY LIVERS OF HYPOPHYSECTOMIZED RATS

Expt.	Rat No.	Strain	Age when sacrificed, days	Wt. when sacrificed, gm.	State	Sacrificed after operation	Incubation medium		Per cent of added C^{14} recovered as	
							Labeled acetate added to flask, micromoles	Unlabeled glucose concentration, mg. per cent	CO_2	Fatty acids
1	7M	L.E.	40	131	Normal	—	10	None	37	4.3
	4M*	L.E.†	56	110	Hyped	16 days	10	None	22	0.5
2	11M	L.E.	40	146	Normal	—	50	None	24	5.4
	12M	L.E.	40	133	Normal	—	50	None	29	6.4
	14F*	L.E.	201	111	Hyped	175 days	50	None	11	0.1
3	15M	L.E.	40	137	Normal	—	90	None	14	3.5
	18M	L.E.	40	132	Normal	—	50	None	13	5.2
	19F	S.D.‡	68	143	Normal	—	50	None	11	8.5
	17M	L.E.	65	119	Hyped	25 days	90	None	11	0.2
	22M	L.E.	56	112	Hyped	14 hours	50	None	8	1.5
	24M	S.D.	68	132	Hyped	24 hours	50	None	26	0.5
	25M	L.E.	41	142	Hyped	24 hours	50	None	26	0.5
4	18M	L.E.	40	132	Normal	—	100	400	17	7.7
	19F	S.D.	68	143	Normal	—	100	400	17	7.7
	16M	L.E.	65	128	Hyped	25 days	100	400	13	0.3
	22M	L.E.	56	112	Hyped	14 hours	100	400	19	1.4
5	20M	L.E.	40	130	Sham-operated	14 hours	50	None	10	4.3
	21F	S.D.	68	151	Sham-operated	14 hours	50	None	11	10.6
	23M	L.E.	41	134	Sham-operated	24 hours	50	None	24	6.7
	20M	L.E.	40	130	Sham-operated	14 hours	100	400	13	3.7
	21F	S.D.	68	151	Sham-operated	14 hours	100	400	13	8.0

* M = male, F = female.
† Long-Evans strain.
‡ Sprague-Dawley strain.

coveries found with the *ad libitum* fed, normal rats were 3.5–8.5%. In the experiments with the sham-operated rats, the recoveries were 4.3–10.6%.

In experiments 1, 2, and 3, acetate was the sole substrate added to the incubation medium. It should be noted that when glucose was added to the incubation medium (Experiments 4 and 5), the fatty acid-C^{14} recoveries observed with the livers of the hypophysectomized rats were still far below those found with livers of normal or sham-operated rats.

DISCUSSION

It is clear, from the data presented here, that the liver of the hypophysectomized rat is not capable of converting added acetate to fatty acids to the same extent as is the liver of the normal rat. A lowered capacity for lipogenesis from added acetate was observed as early as 14 hours and as late as 175 days after complete excision of the pituitary gland.

It is well established, from the work of many investigators, that the level of liver glycogen in *ad libitum*-fed, hypophysectomized rats is normal (Bennett and Evans, 1950). This was also observed in our hypophysectomized rats. The defect in lipogenesis observed here cannot, therefore, be ascribed to a deficient carbohydrate content, *per se*, in the livers of the hypophysectomized rats. Since hepatic lipogenesis does appear to be associated with the extent of glucose utilization (Lyon, Masri and Chaikoff, 1952; Masri, Lyon and Chaikoff, 1952; Hirsch, Baruch and Chaikoff, 1954), it is conceivable that the defect in hepatic lipogenesis observed in the hypophysectomized rat reflects a lowered overall glucose utilization by its liver.

The finding that the $C^{14}O_2$ recoveries were normal in the experiments with the livers of the hypophysectomized rats indicates that there is no obvious impairment in the operative capacity of the tricarboxylic acid cycle in this tissue.

The results presented here are not in agreement with the conclusions drawn by Brady, *et al.* on the effect of hypophysectomy on lipogenesis (1951). On the basis of their findings, it was stated that the "Pituitary gland secretes a principle which can directly or indirectly inhibit hepatic lipogenesis" (Gurin and Crandall, 1951). But this does not hold for the conditions of our experiments in which it would appear that normal hepatic lipogenesis requires some or all of the hormones secreted by the pituitary gland.

SUMMARY

1. The incorporation of the C^{14} of added acetate-1-C^{14} to fatty acids and CO_2 was compared in liver slices prepared from normal and hypophysectomized rats fed, *ad libitum*, an adequate diet.

2. The livers of the hypophysectomized rats lost, to a considerable extent, the capacity to convert acetate carbon to fatty acids. This loss was observed as early as 14 hours and as late as 175 days after hypophysectomy.

3. No appreciable difference in the recoveries of $C^{14}O_2$ was observed in the experiments with livers of normal and hypophysectomized rats.

HANS BARUCH[2] AND I. L. CHAIKOFF

Department of Physiology
University of California School of Medicine
Berkeley

REFERENCES

BARUCH, H. AND I. L. CHAIKOFF: *Proc. Soc. Exp. Biol. and Med.* **86:** 97. 1954.

BENNETT, L. L. AND H. M. EVANS: The Hormones, II, 405, Academic Press, New York, 1950.

BRADY, R. O., F. D. W. LUKENS AND S. GURIN: *J. Biol. Chem.* **193:** 459. 1951.

FELTS, J. M., I. L. CHAIKOFF AND M. J. OSBORN: *J. Biol. Chem.* **191:** 683. 1951.

GURIN, S. AND D. I. CRANDALL: *Ann. Rev. Biochem.* XX: 179. 1951.

HENRIQUES, JR., F. C., G. B. KISTIAKOWSKY, C. MARGNETTI AND W. G. SCHNEIDER: *Ind. Eng. Chem.*, Anal. Ed. **18:** 349. 1946.

HIRSCH, P. F., H. BARUCH AND I. L. CHAIKOFF: *J. Biol. Chem.* **210:** 785. 1954.

KREBS, H. A. AND K. HENSELEIT: *Z. Physiol. Chem.* **210:** 33. 1932.

LYON, I., M. S. MASRI AND I. L. CHAIKOFF: *J. Biol. Chem.* **196:** 25. 1952.

MASRI, M. S., I. LYON AND I. L. CHAIKOFF: *J. Biol. Chem.* **197:** 621. 1952.

[2] Present address: Research Specialties Co., 1148 Walnut Street, Berkeley, California.

MAINTENANCE OF LACTATION IN ADRENALECTOMIZED RATS WITH ALDOSTERONE AND 9α-HALO DERIVATIVES OF HYDROCORTISONE

In this laboratory maintenance of lactation following adrenalectomy in the rat has best been achieved by the administration of cortisone (implantation of 2×11 mg. pellets yielding 0.85 mg./day) together with desoxycorticosterone acetate (DCA) (implantation of 1×50 mg. pellet yielding 0.36 mg./day) (Cowie 1952, and review by Folley 1954). In view of the interesting physiological actions of aldosterone, which shows great DCA-like activity as well as considerable cortisone-like effects (see review by Wettstein and Anner, 1954), it was decided to investigate the ability of this steroid to maintain lactation after adrenalectomy. Moreover in view of recent observations that certain 9α-halo derivatives of hydrocortisone are considerably more active than cortisone in the glyco-

TABLE 1. RESPONSES TO REPLACEMENT THERAPY AFTER ADRENALECTOMY

Groups	No. of mothers	Mean litter-growth indices,* gm. (with S.E.)	Mean total food consumption (gm.) of mothers 5th–12th day inclusive (with S.E.)	Mean percentage change in weight of mothers between 5th–13th days (with S.E.)
Control + sham operation	6	14.5 ± 0.8	276.7 ± 4.5	+4.9 ± 1.3
Adrenalectomy + 0.5 ml. 1% carboxymethylcellulose solution	6	6.2 ± 0.4	175.9 ± 5.4	+2.1 ± 1.2
Adrenalectomy + Hydrocortisone 1 mg./day	6	10.0 ± 0.5	223.4 ± 10.7	+3.2 ± 1.1
Adrenalectomy + Chlorohydrocortisone acetate 100 μg./day	5	13.1 ± 0.5	262.3 ± 8.1	+8.7 ± 2.5
Adrenalectomy + Fluorohydrocortisone acetate 100 μg./day	6	9.9 ± 0.6	222.5 ± 9.4	+7.8 ± 1.6
Adrenalectomy + Aldosterone 50 μg./day	6	9.8 ± 0.7	222.6 ± 10.5	+9.3 ± 0.8

* The litter-growth index of a group of rats is defined as the mean daily gain in weight per litter over the 5-day period from the 6th to the 11th days (Cowie and Folley, 1947 c).

gen-deposition test (Borman and Singer, 1954) and are more effective in maintaining life after adrenalectomy than DCA (Borman and Singer, 1954; Borman, Singer and Numerof, 1954; Leathem and Wolf, 1954), two of these halo-derivatives were included in the experiment.

Owing to the limited quantity of materials available, it has so far only been possible to carry out a pilot experiment, the results of which are recorded below.

MATERIAL AND METHODS

The rats (primiparous females, 4–5 months old) and the general procedures used have been described in previous papers (Cowie and Folley, 1947 a, b). The diet used was the new stock diet for the colony (Cowie and Folley, 1948) to which dried whole milk had been added in the proportion of 375 parts milk powder to 1,000 of diet (by weight). Adrenalectomy was carried out on the 4th day of lactation; the rats in the control groups being subjected to a sham operation. The steroids, 9α-chloro-hydrocortisone acetate, 9α-fluoro-hydrocortisone acetate, and hydrocortisone were administered subcutaneously as crystalline suspensions. A 1% solution of carboxymethylcellulose in saline was used as a suspending agent, the daily dose being contained in 0.5 ml. of this medium. The group of adrenalectomized control rats received daily injections of 0.5 ml. suspending medium. The aldosterone, available to us as an oily solution, was injected subcutaneously. The daily dose being contained in 0.2 ml. oil. All substances were

Received for publication December 26, 1954.

injected once daily, the first injection being given immediately after operation and the final injection on day 12. The rats were killed on day 13 when biochemical investigations were carried out on the livers and mammary glands. The results of these biochemical studies are as yet incomplete and will be reported at a later date.

RESULTS

The litter-growth indices (L.G.I.) (Cowie and Folley, 1947 c) for the various experimental groups, the weight of food eaten by the mothers and their body-weight changes are set out in Table 1. It will be observed that virtually complete maintenance of lactation was obtained with a daily dose of 100 μg. chlorohydrocortisone acetate, the L.G.I. for the group being 13.1 ± 0.5 gm. as against 14.5 ± 0.8 gm. for the controls. The food consumption from the 5th–12th day was maintained at a more or less normal level (262.3 ± 8.1 gm. as against 276.7 ± 4.5 gm.) and the weight gain of the chlorohydrocortisone-acetate-treated group was somewhat greater than that of the control group; the difference, however, is not statistically significant.

Hydrocortisone (1 mg./day), fluorohydrocortisone acetate (100 μg./day), and aldosterone (50 μg./day) all gave partial maintenance of lactation to the same degree. The quantity of food consumed by all these groups was also similar. The gain in weight of the hydrocortisone-treated mothers was, however, significantly less ($P = 0.04$) than the gains observed in the other two groups.

DISCUSSION

The high degree of replacement obtained with chlorohydrocortisone acetate is not surprising in view of the somewhat unique action of this compound which manifests, at least in the rat, both gluco- and mineralo- corticoid-like properties to a very high degree (Borman *et al.*, 1954). The lower replacement value of fluorohydrocortisone acetate is interesting for, although this compound is considerably more active than chlorohydrocortisone acetate in the glycogen deposition test, (Borman and Singer, quoted by Fried and Sabo: 1953, 1954), it has very much less DCA-like activity as deduced from growth and survival tests (Borman et al., 1954). It has been well established that DCA has considerable lactation-maintaining properties in our strain of rat following adrenalectomy (see review by Folley, 1954), and the lower efficiency of fluoro-hydrocortisone acetate may well be due to its low DCA-like activity. Despite the low dose of aldosterone (50 μg./day) and the fact that it was administered in oily solution, the maintenance effect was considerable.

It is planned to carry out further experiments with the above materials, when available, to establish accurately their relative potencies in maintaining lactation in the adrenalectomized rat. Moreover, since Flux (1955) has recently shown that in the adrenalectomized rat the ovary plays a part in the maintenance of lactation, the potency of the above steroids in ovariectomized-adrenalectomized rats requires study.

SUMMARY

Virtually complete maintenance of lactation in the rat following adrenalectomy on the fourth day of lactation was obtained by giving 9α-chlorohydrocortisone acetate (100 μg./day). Partial maintenance was observed with 9α-fluorohydrocortisone acetate (100 μg./day), aldosterone (50 μg./day), or hydrocortisone (1 mg./day).

Acknowledgment

We are indebted to Dr. S. J. Folley, F.R.S. for his advice and interest in this experiment; to Dr. Aleck Borman of the Squibb Institute of Medical Research, New Bruns-

wick; and to Dr. F. Gross of Ciba Ltd., Basle, for gifts to Dr. Folley of the 9α-halogenated compounds and aldosterone, respectively.

A. T. COWIE AND J. S. TINDAL

The National Institute for Research in Dairying
University of Reading
England

REFERENCES

BORMAN, A. AND F. M. SINGER: *Federation Proc.* **13**: 185. 1954.
BORMAN, A., F. M. SINGER AND P. NUMEROF: *Proc. Soc. Exptl. Biol. Med.* **86**: 570. 1954.
COWIE, A. T.: *Endocrinology* **51**: 217. 1952.
COWIE, A. T. AND S. J. FOLLEY: *J. Endocrinol.* **5**: 14. 1947 a.
COWIE, A. T. AND S. J. FOLLEY: *J. Endocrinol.* **5**: 24. 1947 b.
COWIE, A. T. AND S. J. FOLLEY: *J. Endocrinol.* **5**: 9. 1947 c.
COWIE, A. T. AND S. J. FOLLEY: *J. Endocrinol.* **5**: 282. 1948.
FLUX, D. S.: *J. Endocrinol.* **12**: 57. 1955.
FOLLEY, S. J.: Recherches récentes sur la physiologie et la biochimie de la sécrétion lactée. Desoer, Liège; Masson & Cie, Paris, 1954.
FRIED, J. AND E. SABO: *J. Am. Chem. Soc.* **75**: 2273. 1953.
FRIED, J. AND E. SABO: *J. Am. Chem. Soc.* **76**: 1455. 1954.
LEATHEM, J. H. AND R. C. WOLF: *Proc. Soc. Exp. Biol. Med.* **86**: 724. 1954.
WETTSTEIN, A. AND G. ANNER: *Experientia* **10**: 397. 1954.

EFFECT OF ASCORBIC ACID ON THE ADRENAL GLAND AFTER HYPOPHYSECTOMY AND AFTER EXPOSURE TO COLD[1]

The initial report of Dugal and Thérien (1949) that ascorbic acid influences the response of the adrenal gland to stress has been followed by a number of investigations the results of which support the thesis that exogenously administered ascorbic acid modifies the expected responses of the adrenal cortex to cold stress, ACTH administration and hypophysectomy. Thus, Dugal and Thérien (1949) report that ascorbic acid prevented the adrenal hypertrophy observed when rats were placed in the cold for 3 and 244 days. Thérien *et al.* (1949) further state that ascorbic acid administration increases the magnitude of adrenal cholesterol depletion following exposure to cold. Dugal and Thérien (1952) also found that ascorbic acid potentiates the effect of small doses of ACTH on the adrenal weight of hypophysectomized rats. More recently, Khalil (1954) made the interesting observation that ascorbic acid administration prevents almost completely the adrenal atrophy attendant upon hypophysectomy. The present study was undertaken to confirm some of the above observations preparatory to the initiation of a study dealing with the effects of ascorbic acid on adrenal cortical function.

MATERIALS AND METHODS

A. *Hypophysectomy*

1. Male rats of the Sprague-Dawley strain weighing approximately 100 gm. were hypophysectomized by the parapharyngeal approach. Starting the day following operation half of these animals were given a daily intravenous injection of 100 mg. sodium ascorbate[2] in

Received for publication January 4, 1955.
[1] Supported by the Milton Fund of Harvard University.
[2] Nutritional Biochemicals Corp., Cleveland, Ohio.

TABLE 1. EFFECTS OF ASCORBIC ACID ON ADRENAL WEIGHT AND ADRENAL CHOLESTEROL OF HYPOPHYSECTOMIZED RATS

Treatment	No. rats	Adrenal wt. (mg. per 100 gm. body wt.)	Adrenal Chol. (mg. per 100 mg.)
Experiment 1			
Hypox+saline (i.v.)	10	12.34 ±0.71[1]	6.88 ±0.40
Hypox+100 mg. Ascorbic Acid per day (i.v.)	8	11.88 ±0.79	7.97 ±0.36
Experiment 2			
None	5	17.14 ±2.64	3.24 ±0.46
Hypox+saline (i.p.)	15	10.52 ±0.62	7.49 ±0.46
Hypox+150 mg. Ascorbic acid per day (i.p.)	18	10.71 ±0.47	6.53 ±0.37

[1] Mean ±S.E. of mean.

1.0 ml. of 0.9% NaCl. The remainder of the hypophysectomized rats received an equal volume of saline intravenously. The injections were continued for 6 days and the animals sacrificed by ether inhalation on the morning of the 7th day following operation. Their adrenals were removed, cleaned, weighed on a torsion balance and analyzed for total cholesterol (Knobil *et al.*, 1954). Completeness of hypophysectomy was determined by gross inspection of the sella turcica.

2. Male rats of the same strain were hypophysectomized as above. Starting the day of operation, half of these animals were given 3 intraperitoneal injections per day of 50 mg. buffered ascorbic acid[3] in 0.5 ml. saline. The remainder of the hypophysectomized animals received equal volumes of normal saline in lieu of the ascorbic acid. A non-operated control group received no injections. The above regimen was continued for 7 days. The animals were sacrificed on the morning of the 8th day and their adrenals weighed and analyzed as above.

B. *Cold Experiment*

Male rats of the Sprague-Dawley strain weighing approximately 140 gm. were pretreated for 2 days with 3 intraperitoneal injections per day of 50 mg. sodium ascorbate in 0.5 ml. normal saline. They were then placed in a cold room at 4 ±2° C. for 24 hours, the injections being continued during this period. Control animals receiving 0.5 ml. normal saline I.P. 3 times daily were treated as above. A third group of rats receiving no injections was maintained at room temperature (26 ±1° C.). On the morning of the fourth day the animals were sacrificed. Their adrenals were removed, cleaned, weighed on a torsion balance and analyzed for total cholesterol.

RESULTS AND DISCUSSION

The administration of ascorbic acid (given intravenously or intraperitoneally) to hypophysectomized rats did not significantly alter the decrease in adrenal weight or the increase in adrenal cholesterol concentration 7 days after the operation (Table 1).

TABLE 2. EFFECT OF ASCORBIC ACID ON ADRENAL WEIGHT AND ADRENAL CHOLESTEROL FOLLOWING EXPOSURE TO COLD

Treatment	No. rats	Adrenal wt. (mg. per 100 gm.) body wt.)	Adrenal chol. (mg. per 100 mg.)
No Treatment, Room Temperature	6	16.42 ±1.18[1]	3.70 ±0.40
Saline+24 hr. Cold Exposure	10	19.68 ±1.21	2.11 ±0.07
Ascorbic Acid+24 hr. Cold Exposure	8	20.03 ±1.14	1.89 ±0.10

[1] Mean ±S.E. of mean.

[3] Upjohn Company.

The effects of a 24 hour exposure to cold on adrenal weight and adrenal cholesterol concentration were not modified significantly by ascorbic acid treatment (Table 2).

These findings fail to confirm the pertinent reports cited above. The discrepancies between this study and those of Dugal and Thérien (1949), Thérien *et al.* (1949) and Khalil (1954) are difficult to resolve. That these reside in the nature of the ascorbic acid preparation used or the mode of administration is unlikely since both sodium ascorbate and buffered ascorbic acid were utilized in the present investigation with similar results whether given intravenously or intraperitoneally.

SUMMARY

Ascorbic acid administration failed to influence adrenal atrophy and cholesterol concentration following hypophysectomy in rats. Ascorbic acid administration (150 mg. /day) likewise failed to modify adrenal weight and adrenal cholesterol responses in rats exposed to cold for 24 hours. These findings do not confirm previous reports of an influence of ascorbic acid on the alteration of adrenal weight and cholesterol concentration following hypophysectomy and cold stress.

Acknowledgments

The authors are indebted to Elizabeth J. Wilder and Cynthia S. Cook for technical assistance.

ERNEST KNOBIL AND MELVIN J. FREGLY

Department of Physiology
Harvard Medical School
Boston, Massachusetts

REFERENCES

DUGAL, L. P. AND M. THÉRIEN: *Endocrinology* **44:** 420. 1949.

DUGAL, L. P. AND M. THÉRIEN: *Ann. Endocrinol.* **13:** 578. 1952.

KHALIL, H. H.: *Lancet* **1:** 912. 1954.

KNOBIL, E., M. G. HAGNEY, E. J. WILDER AND F. N. BRIGGS: *Proc. Soc. Exp. Biol. and Med.* **87:** 48. 1954.

THÉRIEN, M., J. LEBLANC, O. HEROUX AND L. P. DUGAL: *Canad. J. Research, E.* **27:** 349. 1949.

GLYCOGENOLYTIC EFFECTS OF EPINEPHRINE AND POSTERIOR PITUITARY EXTRACTS ON THE UTERUS AND SKELETAL MUSCLE[1]

Recently, Walaas (1952) reported that large doses of epinephrine failed to decrease the glycogen level of the rat uterus. Since this result seemed inconsistent with the usual glycogenolytic role of epinephrine, a further investigation was made on the action of epinephrine as well as posterior pituitary hormone preparations on rat uterine glycogen.

According to Walaas (1952) only estrogens increase glycogen deposition in the rat uterus but this glycogen, as shown by histochemical techniques, is localized almost exclusively in the myometrium (Walaas, 1952; Bo and Atkinson, 1952, 1953). Therefore, in the experiments to be presented, the alterations in glycogen levels are assumed to occur in the myometrium. Concurrent changes in the glycogen level of a skeletal muscle were also noted.

Received for publication January 12, 1955.

[1] Aided by a grant from the Muscular Dystrophy Associations of America, Inc., and in part by the Sarah Manning Sage and Solon P. Sackett Research Funds of the Department of Zoology of Cornell University.

METHODS

Following the procedure outlined by Walaas (1952), initial uniform glycogen levels in the uteri were obtained by treating spayed female rats with estrogen before administering other hormones. Female rats (180–220 gm.) were spayed; 6–9 days later each was injected with a 50 μg. dose of estradiol benzoate in oil.[2] One day later the rats were starved, and on the following day, tests for glycogenolytic effects of the hormones were made. All rats were in heat 48 hours after estrogen treatment as indicated by vaginal smears.

Epinephrine hydrochloride, Pitocin (Parke-Davis Co.) and a posterior pituitary preparation (Jensen-Salsbery Lab.) were injected in varying amounts in 1 ml. of saline. Epinephrine was injected both subcutaneously and intraperitoneally, while the other preparations were injected only by the latter method. After 1–3 hours, Nembutal was administered, and the *rectus femoris* muscle and uterus were removed, trimmed of extraneous tissue and weighed. The usual methods (Walaas, 1952; Leonard, 1953) were employed to digest the tissue and precipitate the glycogen. Glycogen was determined by the anthrone reagent (Seifter, *et al.*, 1950) and is reported as mg. of glucose per 100 gm. of tissue, wet weight. Data on the uterus is presented on the basis of the whole uterine weight.

RESULTS

The results are summarized in Table 1. Confirmation of the effects of estrogens increasing uterine glycogen (Walaas, 1952; Bo and Atkinson, 1952) and skeletal muscle glycogen (Leonard, 1953) was obtained.

TABLE 1. GLYCOGENOLYTIC EFFECTS OF EPINEPHRINE AND POSTERIOR PITUITARY PREPARATIONS ON UTERUS AND SKELETAL MUSCLE

Treatment			No. of animals	Glycogen (mg./100 g. of tissue)*	
Hormone	Amount	Period (hours)		Uterus	Rectus femoris
Castrate			5	89 ± 6	370 ± 15
Castrate + estrad. ben.	*50 μg. s. c.*		11	314 ± 17	454 ± 24
Epineph.	250 μg. s. c.	1	6	385 ± 21	*189 ± 20*
Epineph.	250 μg. s. c.	3	13	394 ± 23	*74 ± 10*
Epineph.	200 μg. i. p.	1	8	*81 ± 6*	*286 ± 30*
Post. pituitary	0.1 U.S.P.-U. i. p.	1	6	*179 ± 14*	412 ± 31
Post. pituitary	0.5 U.S.P.-U. i. p.	1	6	*110 ± 12*	470 ± 33
Post. pituitary	1.0 U.S.P.-U. i. p.	1	6	*48 ± 7*	452 ± 62
Pitocin	0.1–0.5 I.U. i. p.	1	6	*237 ± 22*	520 ± 16
Pitocin	1.0 I.U. i. p.	1	6	*178 ± 10*	483 ± 20
Pitocin	3–5 I.U. i. p.	1	6	*149 ± 18*	525 ± 20

* All averages are presented with the standard error of the mean. Numbers in italics are significantly different from the estrogen treated controls, $P < 0.02$.

s.c. = Subcutaneous injection; i. p. = Intraperitoneal.

Subcutaneous injections of epinephrine in amounts similar to those employed by Walaas (1952) failed to decrease the glycogen level of the uterus, although the glycogen in the skeletal muscle was markedly lowered. However, intraperitoneal injection of epinephrine decreased the uterine glycogen level in one hour to that observed in the spayed rats not given estrogen. In this instance the decrease in glycogen in the leg muscle was not as great. The increased uterine glycogen level of rats given epinephrine subcutaneously over that of the controls cannot be explained.

The glycogenolytic effects of posterior pituitary preparations on the uterus varied with the dose administered; increasing the dose induced a greater depletion of glycogen. On the basis that 0.1 U.S.P. unit is equivalent to 1 international unit, the glycogenolytic activity of the two preparations was remarkably similar. Neither posterior pituitary preparations lowered the glycogen level of the skeletal muscle.

[2] Estradiol benzoate was generously donated by the Schering Corporation, Bloomfield, New Jersey.

DISCUSSION

It is evident from these experiments that epinephrine will deplete the glycogen of the myometrium if it is administered intraperitoneally. Subcutaneous injection of epinephrine may not have permitted a sufficiently high concentration of hormone to be attained at the uterine level for an effect to be observed. Constriction of uterine blood vessels by the epinephrine itself might have limited the amount of hormone delivered to the organ. It is doubtful that the uterine glycogen concentration was lowered and then restored to the normal level during the 3 hour period, since no decrease was observed when the uterus was examined one hour after epinephrine treatment. On the other hand, intraperitoneal administration would bring a high concentration of the hormone in more direct contact with the uterus. The observations of Shelesnyak (1954) that intra-uterine injections of epinephrine inhibited decidua formation while subcutaneous injections failed to do so, parallel the results obtained in these experiments on uterine glycogen.

It is interesting to compare the effects of subcutaneous versus intraperitoneal injection of epinephrine on the glycogen in the leg muscle. Even after a period of 3 hours, the effect of epinephrine on the skeletal muscle glycogen was far greater than after 1 hour when the hormone was injected intraperitoneally. A prolonged action of epinephrine resulting from a slower absorption at the site of injection might account for the greater effect obtained with subcutaneous administration. A more rapid destruction of epinephrine might be expected after intraperitoneal administration, and a consequent lessening of effect.

The experiments with the posterior pituitary preparations were performed primarily to determine the responsiveness of uterine glycogen to a hormone known to increase uterine contractions. It was observed that on the basis of similar biological units, calculated from the stated potencies, the preparations were equally effective in lowering the glycogen of the uterus. Since Pitocin gave results similar to that of the posterior pituitary preparation, the oxytocic principle might well be the glycogenolytic agent. It is suggested that a measurement of rat uterine glycogenolysis might serve as a standardization method for the oxytocic hormone. Failure of the posterior pituitary hormones to decrease skeletal muscle glycogen is consistent with a previous report (Bischoff and Long, 1931).

SUMMARY

Spayed rats were treated with estrogen to increase the concentration of glycogen in the uterus, primarily in the myometrium. Subcutaneous injections of large doses of epinephrine failed to decrease the uterine glycogen in 1–3 hours, confirming Walaas (1952). Intraperitoneal injections, however, markedly lowered the uterine glycogen indicating this organ is sensitive to epinephrine. Posterior pituitary hormone preparations given intraperitoneally also decreased uterine glycogen in amounts roughly proportional to the dose. Unlike epinephrine, the posterior pituitary hormones failed to decrease glycogen in skeletal muscle.

JACK L. KOSTYO AND SAMUEL L. LEONARD

Department of Zoology
Cornell University
Ithaca, New York

REFERENCES

BISCHOFF, F. AND M. L. LONG: *Am. J. Physiol.* **97**: 215. 1931.
BO, W. J. AND W. B. ATKINSON: *Anat. Rec.* **113**: 91. 1952.
BO, W. J. AND W. B. ATKINSON: *Proc. Soc. Exper. Biol. and Med.* **83**: 405. 1953.
LEONARD, S. L.: *Endocrinology* **53**: 226. 1953.
SEIFTER, S., S. DAYTON, B. NOVIC AND E. MUNTWYLER: *Arch. Biochem.* **25**: 191. 1950.
SHELESNYAK, M. C.: *Endocrinology* **55**: 85. 1954.
WALAAS, O.: *Acta Endocrin.* **10**: 175. 1952.

THE 1955 ANNUAL MEETING

The Thirty-seventh Annual Meeting of The Endocrine Society will be held in the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey, on Thursday, Friday and Saturday, June 2, 3 and 4, 1955.

Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey, is in charge of the local arrangements for the meeting.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The annual dinner is scheduled for Friday, June 3, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately, as the hotels expect to be filled to capacity. Correspond directly with Chalfonte-Haddon Hall, advising time of arrival and departure. Make your reservations now and avoid disappointment.

THE SEVENTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM SPONSORED JOINTLY BY THE ENDOCRINE SOCIETY AND THE INDIANA UNIVERSITY SCHOOL OF MEDICINE AT THE CONTINUATION STUDY CENTER OF THE INDIANA UNIVERSITY MEDICAL CENTER, INDIANAPOLIS, INDIANA— SEPTEMBER 26–OCTOBER 1, 1955

The faculty will consist of twenty-one eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies with emphasis on the clinical aspects, with demonstrations of laboratory tests, presentations of cases, and question and answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program, write Post Graduate Office, Indiana University Medical Center, 1100 West Michigan Street, Indianapolis 7, Indiana. Registration is limited to 100; tuition fee is $100.00. Arrangements for housing accommodations will be made through the Indiana Medical Center.

MEMOIRS OF THE SOCIETY FOR ENDOCRINOLOGY NO. 3: THE TECHNIQUE AND SIGNIFICANCE OF OESTROGEN DETERMINATIONS

The Society for Endocrinology (Great Britain) announces the publication of its third Memoir, reporting the proceedings of a conference held in 1954. It consists of 96 pages of text with numerous illustrations and represents the most up-to-date account of the subject.

It is published by the Cambridge University Press at a price of $3.75. Copies can be obtained in America from the American branch of the Cambridge University Press at 32 East 57th Street, New York 22.